Podocyte molecules in the pancreas and lymphoid tissues and their association to autoimmunity of diabetes

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL PUBLICATIONS

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I Rinta-Valkama J, Palmén T, Lassila M, Holthöfer H. Podocyte-associated proteins FAT, alpha-actinin-4 and filtrin are expressed in Langerhans islets of the pancreas. Mol Cell Biochem, Jul 14, 2006

II Åström E, Rinta-Valkama J, Gylling M, Ahola H, Miettinen A, Timonen T, Holthöfer H. Nephrin in human lymphoid tissues. Cell Mol Life Sci vol. 63(4):498-504, 2006

III Aaltonen P, Rinta-Valkama J, Pätäri A, Tossavainen P, Palmén T, Kulmala P, Knip M, Holthöfer H. Circulating antibodies to nephrin in patients with type 1 diabetes. Nephrol Dial Transplant, Sep 8, 2006

IV Rinta-Valkama J, Aaltonen P, Lassila M, Palmen T, Tossavainen P, Knip M, Holthöfer H. Densin and filtrin in the pancreas and in the kidney, targets for humoral autoimmunity in patients with type 1 diabetes. Diabetes Metab Res Rev, Jun 2, 2006

Publication III has been used as a part of the Ph.D. thesis by Petri Aaltonen.

ABBREVIATIONS

ACTN4	Alpha-actinin-4 gene
AER	Albumin excretion rate
ATP	Adenosine triphosphate
CASK	Calcium/calmodulin-dependent serine protein kinase
CD2AP	CD2-associated protein
cDNA	Complementary deoxyribonucleic acid
CNF	Congenital nephrotic syndrome of the Finnish type
CNS	Central nervous system
cpm	Counts per minute, a scintillation measure unit of radioactivity
CTLA-4	Cytotoxic T lymphocyte antigen-4
DAA	Densin autoantibodies
DN	Diabetic nephropathy
DTT	Dithiothreitol, a chemical reagent
E	Embryonic day
FAA	Filtrin autoantibodies
FAM	6-carboxy-fluorescein
FAT	A member of cadherin superfamily, protocadherin
FITC	Fluorescein isothiocyanate
GAD	Glutamic acid decarboxylase
GADA	Glutamic acid decarboxylase autoantibodies
GFR	Glomerular filtration rate
GLUT1,-2,-4	Glucose transporter proteins
GM-CSF	granulocyte macrophage-colony stimulating factor
HLA	Human leucocyte antigen
IA-2	Islet antigen 2
IA-2A	Islet antigen 2 autoantibodies
IAA	Insulin autoantibodies
ICA	Islet cell autoantibodies
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL-4	Interleukin-4
IQGAP1	IQ motif-containing GTPase-activating protein1
KIRREL	Kin of irregular chiasm C-roughest
LADA	Latent autoimmune diabetes in adults
LAP	Leucine-rich repeats and PSD-95/Dlg-A/ZO-1

LDL	low-density lipoprotein
MAGI	Membrane-associated guanylate kinase inverted
Min	Minute
M-MLV	Moloney murine leukemia virus
MODY	Maturity-onset diabetes of the young
mRNA	Messenger ribonucleic acid
NAA	Nephrin autoantibodies
NEPH1,-2,-3	Nephrin related genes also known as KIRREL-2,-3, Filtrin and NLG
NPHS1	Nephrosis 1, gene encoding nephrin
NPHS2	Nephrosis 2, gene encoding podocin
NS	non-significant
o/n	Overnight
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDZ	PSD-95/Dlg-A/ZO-1, an interaction domain of proteins
PP	Pancreatic polypeptide
PTPN22	Protein tyrosine phosphatase 22
rER	Rough endoplasmic reticulum
RIA	Radioimmunoprecipitation assay
RT-PCR	Reverse transcriptase-polymerase chain reaction
RU	Relative units
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
TBST	Tris-buffered saline with Tween-20
TNF-	Tumor necrosis factor-
TRPC6	Transient receptor potential channel 6
VIC	Fluorescein dye in Taqman Real Time PCR, proprietary name
VNTR	Variable number of tandem repeats
ZO-1	Zonula occludens 1 gene

ABSTRACT

Type 1 diabetes is a disease where the insulin-producing beta cells of the pancreas are destroyed by an autoimmune mechanism. The incidence of type 1 diabetes, as well as the incidence of the diabetic kidney complication, diabetic nephropathy, are increasing worldwide. Nephrin is a crucial molecule for the filtration function of the kidney. It localises in the podocyte foot processes partially forming the interpodocyte final sieve of the filtration barrier, the slit diaphragm. The expression of nephrin is altered in diabetic nephropathy. Recently, nephrin was found from the beta cells of the pancreas as well, which makes this molecule interesting in the context of type 1 diabetes and especially in diabetic nephropathy. In this thesis work, the expression of other podocyte molecules in the beta cells of the pancreas, in addition to nephrin, were deciphered. It was also hypothesised that patients with type 1 diabetes may develop autoantibodies against novel beta cell molecules comparably to the formation of autoantibodies to GAD, IA-2 and insulin. The possible association of such novel autoantibodies with the pathogenesis of diabetic nephropathy was also assessed. Furthermore, expression of nephrin in lymphoid tissues has been suggested, and this issue was more thoroughly deciphered here.

The expression of nephrin in the human lymphoid tissues, and a set of podocyte molecules in the human, mouse and rat pancreas at the gene and protein level were studied by polymerase chain reaction (PCR) -based methods and immunochemical methods. To detect autoantibodies to novel beta cell molecules, specific radioimmunoprecipitation assays were developed. These assays were used to screen a follow-up material of 66 patients with type 1 diabetes and a patient material of 150 diabetic patients with signs of diabetic nephropathy.

Nephrin expression was detected in the lymphoid follicle germinal centres, specifically in the follicular dendritic cells. In addition to the previously reported expression of nephrin in the pancreas, expression of the podocyte molecules, densin,

filtrin, FAT and alpha-actinin-4 were detected in the beta cells. Circulating antibodies to nephrin, densin and filtrin were discovered in a subset of patients with type 1 diabetes. However, no association of these autoantibodies with the pathogenesis of diabetic nephropathy was detected.

In conclusion, the expression of five podocyte molecules in the beta cells of the pancreas suggests some molecular similarities between the two cell types. The novel autoantibodies against shared molecules of the kidney podocytes and the pancreatic beta cells appear to be part of the common autoimmune mechanism in patients with type 1 diabetes. No data suggested that the autoantibodies would be active participants of the kidney injury detected in diabetic nephropathy.

1 Introduction

The molecular structure of the final sieve of the kidney filtration apparatus, the slit diaphragm, has been under intensive study over the last decade. The discovery of nephrin was paramount, since it appears to be the key slit diaphragm protein (Kestila et al. 1998). The discovery of nephrin in the pancreatic beta cells (Palmen et al. 2001) launched the idea whether other podocyte molecules could be expressed in the beta cells of the pancreas as well. In addition to nephrin expression in the kidney podocytes and the beta cells of the pancreas, preliminary data have suggested that nephrin expression occurs in the lymphoid tissues, specifically in rat spleen (Ahola et al. 1999) and mouse thymus (Liu et al. 2001). Furthermore, many beta cell molecules have been shown to be immunogenic and autoantibodies against these molecules are detected in patients with type 1 diabetes (Palmer et al. 1983; Baekkeskov et al. 1990; Lan et al. 1996).

The purpose of this thesis was to explore the possible expression of podocyte molecules in the pancreas and the lymphoid tissues. In addition, antigen-specific radioimmunoprecipitation assays (RIA) were developed to decipher if autoantibodies against nephrin and other novel beta cell molecules are formed in patients with type 1 diabetes. The possibility whether these novel autoantibodies could participate in the development of diabetic nephropathy was also assessed.

2 Review of the literature

2.1 Pancreas

2.1.1 Structure and function

The pancreas is an organ located in the abdominal cavity and it has been descriptively divided into a head, a body and a tail. The main pancreatic duct runs the length of the gland and empties into the upper duodenum, together with the duct from the liver and gallbladder (Figure 1). The exocrine and endocrine functions of the pancreas are carried out by two distinct subunits. The exocrine component includes acinar cells that secrete digestive enzymes (proteolytic endo- and exopeptidases, amylolytic enzymes, lipases and nucleolytic enzymes) and a highly branched ductal system of centroacinar and ductal cells that transport these digestive enzymes into the intestine. The intercalated duct cells of the ductal system secrete bicarbonate which neutralises the acidity of the chyme coming from the stomach to establish an optimal pH for the activity of digestive enzymes. The exocrine cells make up the majority of the pancreas, representing 95-99% of the mature pancreas (Ross et al. 2003a).

The endocrine pancreas consists of about 1 million islets of Langerhans comprising approximately 1-2% of the volume of the pancreas. The Langerhans islets are small spheroid clusters of cells, distributed throughout the organ. They vary considerably in size, ranging from tens of cells to several thousand cells. The islets are richly vascularised by a network of fenestrated capillaries. The endocrine portion is composed of five cell types, alpha, beta, delta, PP (pancreatic polypeptide) and ghrelin cells. Proportions of the main cell types of the endocrine pancreas and blood glucose homeostasis regulating hormones secreted by them are described in Table 1.

Cell type	Proportion of the islet in adult	Hormones secreted	Main function
Alpha cell	~30%	glucagon	blood glucose ↑
Beta cell	~60%	insulin amylin	blood glucose ↓ insulin action modulation
Delta cell	5-10%	somatostatin	insulin and glucagon ↓
PP cell	~1%	pancreatic polypeptide	pancreatic secretion ↓
Ghrelin cell	~1%	ghrelin	insulin ↓

Table 1. *Cell types of the endocrine pancreas and proportions of them in human pancreatic Langerhans islet. The main functions of the secreted hormones are indicated (Westermark et al. 1987; Wierup et al. 2002; Cabrera et al. 2006).*

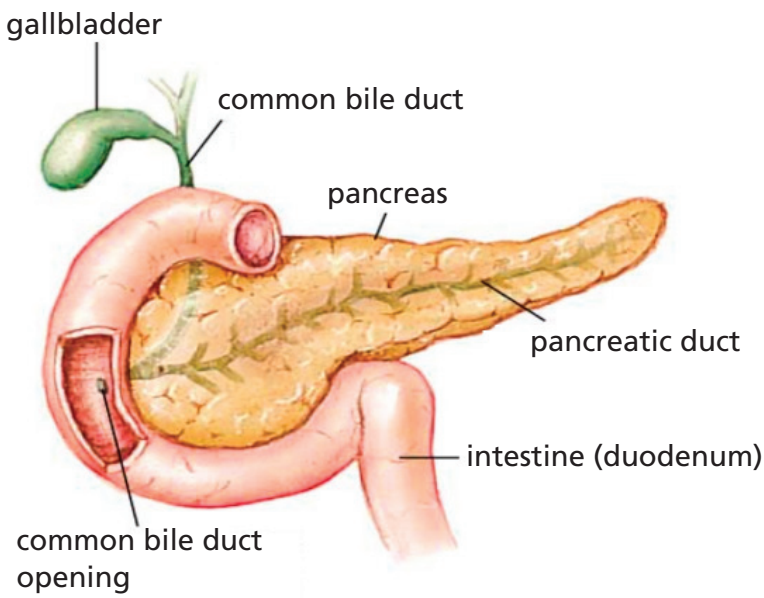

Figure 1. *Structure of the human pancreas. Localisation of the pancreas next to the intestine and the gallbladder. The main pancreatic duct runs through the middle line of the pancreas (Modified from a picture by Houghton Mifflin Company, www.yourdictionary.com).*

2.1.2 Development

In the mouse, at embryonic day (E) 9.5 dorsal and ventral pancreatic buds bulge out from the foregut endodermal epithelium. This is followed by branching morphogenesis by E12.5 and subsequent fusing of the dorsal and ventral pancreata into a single organ on E14. Exocrine pancreas differentiates from the ductal epithelium to acini and ducts during E14.5 and E15.5. After E14 the endocrine cells, scattered as single cells within the ductal epithelium, proliferate extensively and on E16 they start to form islet-like clusters. The islet of Langerhans is fully formed on E19 but islet maturation and remodelling continues until 2-3 weeks after birth. The first endocrine cells detected, at the pancreatic bud stage E9.5, express glucagon and pancreatic polypeptide. Subsequently, the cells divide into distinct lineages of alpha, beta and PP cells. At E14 the somatostatin expressing delta cells arise. Signals from the mesoderm, notochord and endothelial cells are important for the orchestrated development of the pancreas (Habener et al. 2005; Hill 2005).

2.1.3 Blood glucose control

The main role of the hormones secreted by the pancreatic beta cells is to maintain glucose homeostasis in the body. Insulin hormone is a 6-kDa polypeptide consisting of two chains, the A and B chains, which are linked by disulfide bonds. The main target tissues of insulin are the liver, skeletal muscle and adipose tissue. Insulin stimulates the uptake of glucose from the circulation mainly through the glucose transporter GLUT4 and activates glucose storage by activating glycogen synthesis. In addition to glucose metabolism, insulin takes part in lipid and protein metabolism; it inhibits lipase activity in adipose cells, accelerates amino acid uptake into cells and inhibits protein catabolism. Absence of insulin leads to elevated blood glucose levels by decreased uptake in the target tissues. Glucagon, secreted by the alpha cells, has mainly the opposite effects to insulin. Glucagon secretion is stimulated by the reduction in blood glucose concentration. It excites the release of glucose into the circulation, gluconeogenesis, breakdown of glycogen in the liver, and mobilises fats from the adipose cells (Ross et al. 2003a).

In rodents, there are two insulin genes (Lomedico et al. 1979; Wentworth et al. 1986) while in man, insulin is encoded by a single gene INS which is located on the short arm of chromosome 11 (11p15.5) (Harper et al. 1981). Insulin is derived from a large single-chain precursor, preproinsulin, which includes a signal peptide, the A and B chains and a third chain that connects (C-peptide) the A and B chains. Preproinsulin is synthesised in the rough endoplasmic reticulum (rER) and is further processed in the Golgi apparatus. The signal sequence is removed in the rER forming proinsulin which is subsequently transferred to Golgi apparatus. In the Golgi the proinsulin is enclosed in a clathrin-coated secretory vesicles and C-peptide is removed from the linked insulin A and B chains. The vesicles lose their clathrin coat and mature intracellular granules containing insulin form. When glucose enters the beta cell through glucose transporter 2 (GLUT-2), it increases glucose metabolism. The elevation in the adenosine triphosphate (ATP) concentration closes the K_{ATP} channels causing depolarisation of the plasma membrane, activation of Ca^{2+} channels, Ca^{2+} entry and fusion of the secretory vesicles with the plasma membrane. Finally, insulin is carried via efferent capillaries to systemic circulation (Koster et al. 2005).

2.2 Kidney

The kidneys are paired bean-shaped organs located on both sides of the spine in the posterior abdominal cavity. The human kidney is approximately 10 cm long and 6.5 cm wide. The main function of the kidneys is the filtration of blood plasma and excretion of metabolic waste products into the urine. In addition, the kidneys maintain the water, electrolyte and pH balance of the body and secrete hormones which regulate blood pressure and red blood cell formation. Each kidney contains approximately 500 000-1 million nephrons, the functional units of the kidney. Each nephron is composed of a compact tuft of capillary loops (glomerulus) surrounded by the Bowman's capsule and an associated tubulus with a distinct proximal and distal part. The glomerulus is responsible for the formation of the primary urine and the tubular system reabsorbs valuable substances, such as water, important proteins and glucose, back into the circulation. The final urine then flows from the tubules through the collecting duct to the renal pelvis and finally to the urinary bladder (Kierszenbaum 2002a).

2.2.1 Glomerulus, podocyte and slit diaphragm

The glomerulus is composed of mesangial cells which provide structural support for the glomerular capillaries and which produce the mesangial matrix, the Bowman's capsule where the primary urine is sieved and the three-layered glomerular filtration barrier through which the primary urine is formed. The filtration has been shown to be selective for charge and size (Brenner et al. 1978). The layers of the filtration barrier include fenestrated endothelium, glomerular basement membrane and podocytes, the highly specialised visceral epithelial cells. The podocytes are polarised octopus-like cells with long primary processes and small secondary foot processes. The interdigitating foot processes from neighbouring podocytes completely surrounds the glomerular capillaries and a zipper-like intercellular junction structure, a slit diaphragm, separates the foot processes from each other (Rodewald et al. 1974). The slit diaphragm is the final sieve of the glomerulus allowing passage of small molecules but preventing leakage of large molecules to urine. The molecular composition of the slit diaphragm was unknown for a long time, but during the last two decades the molecules have been partly characterised. The currently known molecules of the slit diaphragm area are listed in Table 2. The slit diaphragm has molecular and structural characteristics of tight junctions (Schnabel et al. 1990; Harhaj et al. 2004) and adherens junctions (Reiser et al. 2000; Inoue et al. 2001; Ciani et al. 2003; Lehtonen et al. 2005), and therefore the slit diaphragm can be defined as a modified adherens junction.

Molecule	Reference
ZO-1	(Schnabel et al. 1990)
Synaptopodin	(Mundel et al. 1997)
Nephrin	(Kestila et al. 1998)
CD2AP	(Shih et al. 1999)
P-cadherin	(Reiser et al. 2000)
Catenins	(Reiser et al. 2000)
Podocin	(Boute et al. 2000)
Alpha-actinin-4	(Kaplan et al. 2000)
FAT	(Inoue et al. 2001)
NEPH1	(Donoviel et al. 2001)
NEPH2	(Sellin et al. 2003)
Filtrin	(Ihalmo et al. 2003)
Densin	(Ahola et al. 2003)
Fyn	(Verma et al. 2003)
CASK	(Lehtonen et al. 2004)
MAGI-2	(Lehtonen et al. 2005)
IQGAP1	(Lehtonen et al. 2005)
Spectrins	(Lehtonen et al. 2005)
TRPC6	(Reiser et al. 2005)
MAGI-1	(Hirabayashi et al. 2005)
Nck	(Jones et al. 2006)

Table 2. *The slit diaphragm molecules arranged by the timing of their discovery in the podocytes. CASK, calcium/calmodulin-dependent serine protein kinase; CD2AP, CD2-associated protein; IQGAP1, IQ motif-containing GTPase-activating protein1; MAGI, membrane-associated guanylate kinase inverted; TRPC6, canonical transient receptor potential 6; ZO-1, zonula occludens-1.*

2.2.2 Nephrin

In the interpodocyte slit diaphragm, nephrin was the first molecule shown to be essential for the proper structure and function of the filtration slit. The gene encoding nephrin, NPHS1, was found by positional cloning by Kestilä and colleagues when they searched for the gene defective in patients with congenital nephrotic syndrome of the Finnish type (CNF) (Kestila et al. 1998). CNF is a serious monogenic disease manifesting with severe proteinuria already in utero and subsequent kidney failure due to podocyte injury. The NPHS1 gene is located in the chromosome locus 19q13.1 (Kestila et al. 1994; Mannikko et al. 1995). Nephrin protein consists of one fibronectin-like domain, eight immunoglobulin (Ig)-like domains, a transmembrane section and a short intracellular domain. It has been included into the immunoglobulin superfamily on the basis of its immunoglobulin-like domains (Kestila et al. 1998). It was originally postulated that the expression of nephrin is restricted to the kidney glomerulus, but now its expression has been shown in the pancreas (Palmen et al. 2001; Zanone et al. 2005), the brain (Putaala et al. 2001) and the testis (Liu et al. 2001). In addition, nephrin expression has been suggested to occur in the placenta (Beall et al. 2005), the spleen (Ahola et al. 1999) and the thymus (Liu et al. 2001). The calculated molecular weight of nephrin is ~135 kDa (Kestila et al. 1998) but it presents with a molecular weight of 165-185 kDa in different tissues (Ahola et al. 1999; Holzman et al. 1999; Palmen et al. 2001). The variance in molecular weight has been suggested to be due to differential glycosylation (Yan et al. 2002). There appears to be tissue-specific differences in the regulation of nephrin expression (Moeller et al. 2000; Wong et al. 2000; Eremina et al. 2002; Moeller et al. 2002; Beltcheva et al. 2003). The functions of nephrin in these extrarenal tissues remain poorly defined.

In the kidney, nephrin is an essential structural component of the slit diaphragm zipper-like cell-cell contact and it has been suggested that nephrin forms this interpodocyte bridge by a homotypic interaction (Kestila et al. 1998; Ruotsalainen et al. 1999; Tryggvason et al. 1999). Furthermore, the extracellular parts of nephrin have been shown to form a heterodimeric interaction with the members of the NEPH-protein family (Barletta et al. 2003; Gerke et al. 2005). Nephrin has been suggested to be linked to the actin cytoskeleton

through adaptor molecules, including the CD2-associated protein (CD2AP) (Yuan et al. 2002) and Nck (Jones et al. 2006; Verma et al. 2006). In addition to the central role in the zipper-like cell-cell contact, nephrin is essential for the proper cytoskeletal organisation of the podocytes. This is evident in CNF children lacking nephrin who present with fused (effaced) foot processes and absent slit diaphragms (Huttunen et al. 1980; Suren et al. 1993). A similar phenotype is seen in nephrin knock-out animal kidneys (Putaala et al. 2001; Rantanen et al. 2002).

Typically members of the immunoglobulin superfamily are involved in cell adhesion and signaling (Juliano 2002). The cytoplasmic tail of nephrin contains several tyrosine residues and thus has the ability to be phosphorylated (Simons et al. 2001). Indeed, many studies recently have shown the important role of nephrin in initiating the outside-in signaling in podocytes (Huber et al. 2001; Simons et al. 2001; Huber et al. 2003a; Lahdenpera et al. 2003; Verma et al. 2003; Li et al. 2004; Verma et al. 2006). The other established molecules of the slit diaphragm, podocin (Huber et al. 2001) and NEPH1 (Huber et al. 2003b), have also been implicated in signaling processes. Nephrin and NEPH1 mediated signaling appears to be needed for the proper organisation of the slit diaphragm cell junction and prevention of apoptosis of the podocytes (Huber et al. 2005).

2.2.3 Densin

Densin is a protein originally thought to be brain-specific, but recently Ahola and colleagues demonstrated that densin is found in the kidney glomerular podocytes where it localises in the slit diaphragm and appears to interact with nephrin (Ahola et al. 2003). In the brain, densin is an abundant protein found in postsynaptic densities of neurons where it is detected with the molecular weight of 180 kDa (Apperson et al. 1996; Walikonis et al. 2001). However, in kidney glomerulus lysates densin appeared as a 210 kDa band which suggests different post-translational modification or alternative splicing (Strack et al. 2000; Ahola et al. 2003; Wang et al. 2003).

Densin belongs to the LAP (leucine-rich repeats [LRR] and PDZ [PSD-95/Dlg/ZO-1] domains) protein family. More specifically the sequence of densin contains 17 leucine-rich repeats, a putative

transmembrane domain and a PDZ domain. This arrangement of domains is similar to several cell adhesion molecules (Apperson et al. 1996; Walikonis et al. 2001). However, the argument that densin is a transmembrane protein has been questioned lately since densin is not accessible to biotinylation, a method used to label extracellular parts of membrane proteins (Izawa et al. 2002; Quitsch et al. 2005). In the light of the recent findings, densin seems to be targeted to the inner leaflet of the plasma membrane by the leucine-rich region and to anchor there, but not to traverse the plasma membrane (Quitsch et al. 2005). In addition to cell-cell contacts, other proposed functions of densin include signal transduction (Quitsch et al. 2005) and maintaining cell polarity (Bilder et al. 2000). In podocytes it has been suggested that densin could have a role in maintaining the apical-basal polarity (Ahola et al. 2003). Interestingly, a recent report shows that densin is expressed in the Sertoli cells of the testis and the authors suggest that densin could be an adherens junction protein between Sertoli cells and developing germ cells (Lassila et al 2006).

2.2.4 Filtrin

Filtrin/NEPH3/KIRREL2 is a NEPH-protein family member which is expressed in the beta cells of the pancreas (Sun et al. 2003) and in the slit diaphragm (Ihalmo et al. Unpublished results) between the kidney glomerular podocytes (Ihalmo et al. 2003). Generally, the tissue distribution of filtrin appears to be quite restricted since in northern dot blot analysis comprising 76 human tissues, expression was detected only in the pancreas, kidney and lymph nodes (Ihalmo et al. 2003; Sun et al. 2003). In addition, filtrin appears to be expressed in the central nervous system (CNS) as well, since a filtrin EST clone has been found in a brain expression library (Sun et al. 2003) and, recently, filtrin was shown to be expressed in the developing CNS (Minaki et al. 2005). At least four splicing variants have been suggested for filtrin and they appear to have different tissue distribution. Two of them were found from pancreatic complementary DNA (cDNA), one from fetal brain and one from a retinoblastoma cell line (Sun et al. 2003).

Filtrin has significant sequence homology and structural similarity with several proteins including the other two NEPH-family members NEPH1, NEPH2, mammalian KIRREL (kin of irregular chiasm C-roughest) and nephrin (Ihalmo et al. 2003; Sellin et al. 2003; Sun et al. 2003; Gerke et al. 2005). Filtrin contains five immunoglobulin domains, one transmembrane domain and an intracellular domain (Ihalmo et al. 2003) and, as a member of the immunoglobulin superfamily, it has properties of cell adhesion molecules (Minaki et al. 2005). The extracellular parts of NEPH1 (Barletta et al. 2003) and NEPH2 (Gerke et al. 2005) interact with nephrin, and, in addition, all the NEPH family members have been reported to interact with podocin with their intracellular sections (Sellin et al. 2003). Filtrin homologues NEPH1 and NEPH2 have been shown to have a signaling function (Sellin et al. 2003). Filtrin has been proposed to have comparable function, since it has a potential phosphorylation site in its cytoplasmic domain (Ihalmo et al. 2003) and has high homology and structural similarity with NEPH1 and NEPH2. Filtrin/NEPH3/KIRREL2 is located in the human genome in chromosome 19q13.1 next to the nephrin (NPHS1) gene and interestingly, they are transcribed in opposite directions (Ihalmo et al. 2003). The functional peculiarities of this bidirectional promoter and regulatory area have not yet been characterised.

2.2.5 Other slit diaphragm-associated proteins

Nephrin and the proteins in its vicinity form an important functional complex linking the slit diaphragm, a cell junction, to the actin cytoskeleton. Indeed, the actin cytoskeleton of the podocyte foot processes is directly linked to the slit diaphragm protein complex (Huber et al. 2005). The adaptor molecules CD2AP (Welsch et al. 2001; Palmen et al. 2002) and ZO-1 (Huber et al. 2003b) mediate a direct or indirect connection between nephrin and the NEPH-family members to the actin cytoskeleton. Nephrin has also been suggested to form a complex with adherens junction proteins P-cadherin and p120 catenin which are linked to the cytoskeleton via the adaptor proteins ZO-1 and CD2AP as well (Lehtonen et al. 2004). In addition, densin binds to alpha-actinin-4, the actin bundling protein, suggesting another connection between slit diaphragm protein complex and actin network (Walikonis et al. 2001). The large (~500 kDa) protocadherin FAT, also located

in the slit diaphragm, has been suggested to have a role in cell adhesion, signaling (Ciani et al. 2003) and actin dynamics in the podocytes (Moeller et al. 2004). A schematic presentation of the proteins in the slit diaphragm area is shown in Figure 2.

Interference of the slit diaphragm protein complex has been shown to result in disruption of the podocyte architecture, effacement of the podocyte foot processes and proteinuria. Mutations in the podocin gene (NPHS2) in humans causes autosomal recessive steroid-resistant nephrotic syndrome which rapidly proceeds to end-stage renal disease (Boute et al. 2000), and mutations in alpha-actinin-4 gene (ACTN4) result in familial focal segmental glomerulosclerosis (Kaplan et al. 2000). Furthermore, in mice, Neph1 knock-out (Donoviel et al. 2001) caused proteinuria and early postnatal death, CD2AP knock-out resulted in renal failure by seven weeks of age (Shih et al. 1999) and knock-out of alpha-actinin-4 (Kos et al. 2003) caused severe glomerular disease and premature death. Fat1 knock-out presented as loss of slit diaphragms, severe podocyte foot process effacement and perinatal death (Ciani et al. 2003). The serious defects in these human diseases and animal models illustrate the crucial importance of these molecules in the proper filtration function of the kidney.

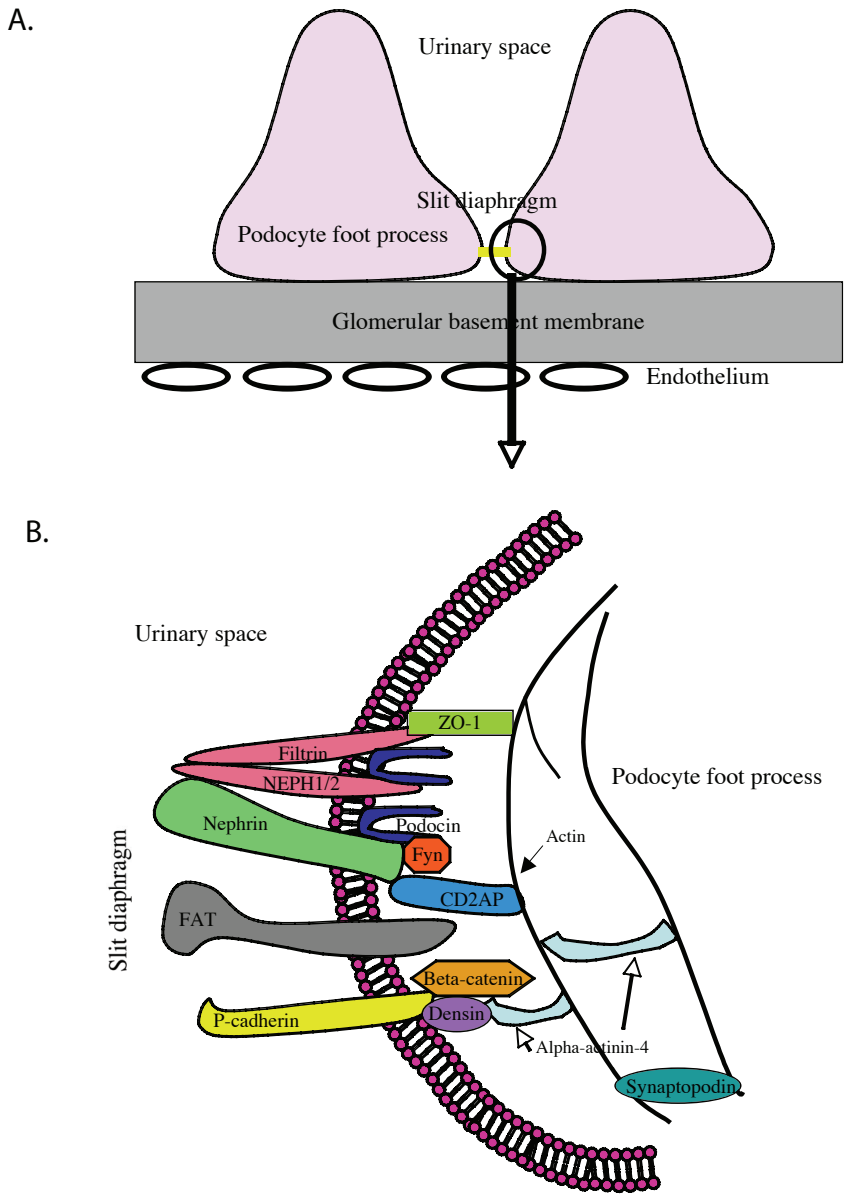

Figure 2. *Schematic drawing of the podocyte foot processes and the slit diaphragm area. Podocyte foot processes are attached to the underlying glomerular basement membrane and interconnected by a structure called the slit diaphragm (A). Closer view of the slit diaphragm including some of the established molecules and densin and filtrin which has been suggested to be localised at the filtration slit (Ahola et al. 2003; Ihalmo et al. 2003) (B).*

2.3 Lymphoid tissues

Lymphoid tissues mainly consist of dense accumulations of lymphocytes and they are also the sites of proliferation and differentiation of these cells. The lymphoid organs are divided into two groups, the primary lymphoid organs including the bone marrow and the thymus, and secondary lymphoid organs like the lymph nodes, the spleen and the tonsils. Lymphoid tissues are typically located at sites that provide a possible route of entry of pathogens and sites that are liable to infections. The main function of the lymphoid tissues and cells is to protect the body against invading pathogens. The immune system has the ability to distinguish the body's own tissues and molecules from external antigens, but when this capability is aberrant it can result in a variety of autoimmune diseases. Mainly, the lymphoid system comprises of two types of cells, the lymphocytes and the accessory cells. Lymphocytes include B cells, helper T cells (CD4+), cytotoxic T cells (CD8+), regulatory T cells and natural killer cells, and the accessory cells include macrophages, dendritic cells and follicular dendritic cells (Kierszenbaum 2002b).

2.3.1 Thymus

The thymus is an organ situated in the upper parts of the thorax, behind the upper four costal cartilages. The size of the thymus changes in the course of life, in an adult it weights about 10 g. The thymus is formed from incomplete lobules each of which are constructed of an outer darker zone, the cortex and a lighter central zone, the medulla. The thymus is necessary for the development of the recirculating pool of small, long-lived T lymphocytes. These cells are mainly responsible for the cell-mediated part of the immune response (Kierszenbaum 2002b).

T lymphocyte progenitors formed in the bone marrow enter the thymus as immature thymocytes and in the thymus they mature into immunocompetent T cells. Most T lymphocyte development takes place in the cortex. First, the thymocytes proliferate and mature from double-negative T cells (CD4-, CD8-coreceptor negative) into double-positive T cells (CD4+, CD8+). This is followed by positive selection, a process where the double-positive cells are activated with cortical epithelial cells to single-positive T cells (either CD4+ or CD8+). This process is mediated by the interaction between

human leucocyte antigen (HLA) class I or class II molecules in the cortical epithelial cells and the T cell receptors in the T cells. The T cell receptor has to recognise the own HLA molecule with sufficient and appropriate affinity. Subsequently, the process called negative selection eliminates T cells which bind with too high affinity to own HLA molecules and the T cells which recognise self-antigens. Negative selection is mostly performed in the thymic medulla, which has an important function in the development of self-tolerance, the tolerance to peripheral self-antigens. A small minority of thymic cells (1-3%), the medullary thymic epithelial cells, express small amounts of a wide variety of tissue-restricted self-antigens, eg. proinsulin, which results in tolerance of these antigens when the cells of the lymphoid system encounter these antigens elsewhere in the body (Derbinski et al. 2005; Wing et al. 2006). Only about 5% of the original thymocytes survive the selection processes. Finally, the T cells migrate from the thymic medulla into the blood stream and further to the T lymphocyte areas of the secondary lymphoid organs. Since the function of the thymus is to produce lymphocytes for the other lymphoid tissues, it is classified as a primary lymphoid organ.

2.3.2 Lymph nodes

Lymph nodes are small, flattened, oval shaped organs, which are located in the course of the collecting lymph vessels. Their size varies from a few millimeters to more than two centimeters. The role of lymph nodes is to defend against lymph-borne micro-organisms (bacteria, viruses and parasites) and maintain and produce antigen specific B cells. In lymph nodes, the B- and T cells, follicular dendritic cells, macrophages and reticular cells are found. The lymph enters the lymph node through afferent lymph vessels and subsequently flows into the subcapsular space. The lymph flow continues thereafter through sinuses towards the efferent lymph vessels, which exit from the hilus of the lymph node (Kierszenbaum 2002b).

The lymph node is divided into a cortex and a medulla. In the outer cortex of the lymph node the B- and T lymphocytes are organised into spherical masses, the lymphoid follicles (Figure 3). As a result of antigen stimulation, the antigen-specific B cells and antigen-activated helper T cells interact outside the lymphoid follicles. This results in the activation of B cells, and the activated B cells subsequently

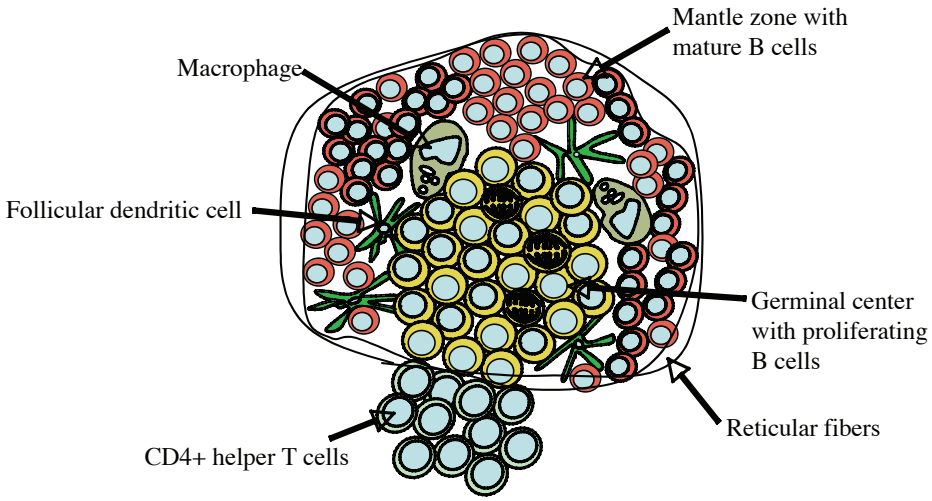

Figure 3. *Structure of the lymphoid follicle, modified from Kierszenbaum et al (Kierszenbaum 2002b). The lymphoid follicle comprises of the germinal centre and the mantle zone. CD4+ helper T cells activate B cells by secreting lymphokines such as IL-4 and IL-5. The B cells then proliferate in the germinal centre. The mature B cells establish a contact with follicular dendritic cells, which display intact antigens on their cell surface. Subsequently the mature B cells accumulate in the mantle zone. Macrophages phagocytose the apoptotic B cells. Stroma of reticular fibers surrounds the lymphoid follicle.*

migrate to the nearby follicles forming structures called germinal centres. The germinal centres are the sites of B cell proliferation and maturation and the helper T cells provide proliferative signals necessary for B cell expansion. Follicular dendritic cells form a close contact with the maturing B cells and they introduce intact antigen-antibody complexes for recognition by these cells. This is a process vital for the rescue of the mature B cells from apoptosis (Cozine et al. 2005).

2.3.3 Spleen

The spleen is the largest secondary lymphoid organ in the body, the average size being about 10 centimeters in diameter. It is located in the upper-left part of the abdomen below the diaphragm. Unlike the lymph nodes, the spleen is connected to the blood stream. The spleen clears the blood of aged blood cells and foreign particles and is the site of immune reactions to blood-borne antigens (Kierszenbaum 2002b).

Most of the spleen is composed of the macrophage- and red blood cell-occupied tissue called the red pulp. Its role is to remove the damaged and aged red blood cells and micro-organisms from blood circulation. The red pulp is also a storage place for red blood cells. Within the red pulp there are small, oval or rounded white areas, the white pulp, which is formed of lymphoid tissue. The white pulp is similar in cell composition to lymph nodes and it reacts to microorganisms and other foreign molecules that reach the bloodstream. Germinal centres in the white pulp are sites of B lymphocyte proliferation (Kierszenbaum 2002b).

2.3.4 Tonsils

Tonsils are accumulations of lymphatic tissues found at the openings of the respiratory and digestive tracts. Tonsils are at their largest during childhood and later in life the size is reduced. Palatine tonsils are situated in the lateral wall of the oropharynx, lingual tonsils are located in the lamina propria at the root of the tongue, and pharyngeal tonsils or adenoids are situated at the upper posterior part of the throat (nasopharynx). These three types of tonsils are collectively named as the Waldeyer's ring. These are the sites where an organism comes into contact with various foreign agents in the air and in food. Thus, the tonsils act as part of the immune system to help to protect the body against infection, particularly against pharyngeal and upper respiratory tract infections (Nave et al. 2001; Ross et al. 2003b).

The tonsils have efferent lymph vessels but the afferent lymph vessels are lacking. Hence the antigen exposure occurs across a specialised epithelium, the lymphoepithelium, covering the tonsils. The epithelium of the palatine and lingual tonsils forms deep crypts in the lymphoid tissue, which results in an increase of the surface area. This facilitates the contact of antigens with the immune cells. Tonsillar lymphoid tissue consist mainly of B lymphocytes, which, similarly to lymph nodes, form lymphoid follicles with follicular dendritic cells and T lymphocytes. Other areas are occupied by T lymphocytes, activated B lymphocytes and other cells of the immune system (Nave et al. 2001; Ross et al. 2003b).

2.4 Diabetes

Diabetes is a chronic condition which occurs when the pancreas does not produce enough insulin or when the body can not effectively use the insulin it produces. This results in an inbalance of glucose homeostasis in the body and in a rise of blood glucose level. There are two main forms of diabetes, type 1 and type 2. Type 1 diabetes is an autoimmune disease typically diagnosed at a young age, whereas type 2 diabetes can be characterised as a lifestyle disease of middle-aged or older people. According to the World Health Organisation the number of all diabetes patients in the year 2000 was 171 million and the number is estimated to double in the next thirty years (www.who.int/diabetes/facts/world_figures/en/).

2.4.1 Type 1 diabetes

Type 1 diabetes is a chronic inflammatory disease where insulin producing beta cells in the Langerhans islets of the pancreas are destroyed by an autoimmune mechanism. This eventually leads to total insulin deficiency and as a consequence, inability of the cells to utilise blood glucose. Both cellular and humoral immune responses have been observed in the disease process. The current view of the disease progression is that antigen presenting cells (dendritic cells), which have picked up soluble beta cell proteins or proteins derived from apoptotic or necrotic beta cells, present these peptides to T cells in the pancreatic lymph nodes. The activated T cells then migrate to the pancreas (insulitis) and recognise the respective epitopes there. This results in a cell mediated response when macrophages and autoreactive T cells damage and kill the beta cells. Intracellular proteins are released from the damaged islet cells, B lymphocytes recognise these as foreign, and finally, start producing antibodies against these antigens. At the time of diagnosis, there is end-stage insulitis and most (80-90%) of the beta cells are lost (Mathis et al. 2001; Daneman 2006). In the first phases, the symptoms of type 1 diabetes are usually very sudden and they include a high level of glucose in the blood and urine, excessive thirst, frequent urination, hunger and weight loss, weakness, blurred vision, nausea and irritability. Patients with type 1 diabetes will not survive without constant insulin replacement therapy.

The incidence of type 1 diabetes has been increasing considerably during the last decades, especially

among young children in developed countries. The increase in the incidence has been about 2-5% per year worldwide (Daneman 2006). Finland is the country with the highest incidence of type 1 diabetes (Karvonen et al. 2000) and the current estimation of the number of patients is more than 30 000 (www.diabetes.fi).

2.4.2 Type 2 diabetes

Type 2 diabetes is a lifestyle-dependent disease associated with the metabolic syndrome. The typical features of the metabolic syndrome in addition to hyperglycemia are obesity (especially abdominal obesity), hypertension, dyslipidemia (high LDL (low-density lipoprotein), cholesterol and triglyserides) and microalbuminuria. They are all important cardiovascular risk factors (Alberti et al. 1998).

Type 2 diabetes presents with reduced insulin sensitivity in insulin sensitive tissues, such as the liver and the adipose tissue, as well as with defective insulin secretion by the beta cells. In the early stages of the disease the predominant abnormality is reduced insulin sensitivity, characterised by elevated levels of insulin and glucose in the blood. The symptoms of type 2 diabetes are similar to type 1 diabetes but they develop more gradually, and some people with type 2 diabetes are even asymptomatic. Patients with incipient type 2 diabetes are treated by controlling of weight and diet as well as by increased physical activity. Pharmaceuticals are needed in most cases for blood glucose management: drugs to enhance the insulin sensitivity in tissues and insulin administration in the cases with impaired insulin secretion.

According to the International Diabetes Federation there are estimated to be at least 140 million people in the world with type 2 diabetes (www.idf.org). The number of diagnosed type 2 diabetic patients in Finland is about 200 000. Additionally, many people are unaware of having the disease (Reunanen 2004).

2.4.3 LADA

LADA (latent autoimmune diabetes in adults) is phenotypically categorised as a subtype of type 2 diabetes but it appears to be a type 1 and type 2 diabetes intermediate. It has also been categorised as slowly developing type 1 diabetes. Typical features of LADA are that the patients do not need insulin replacement therapy at the time of diagnosis and at

least during the first 6 months and insulin deficiency develops during the first few years. Thus, beta cell failure is slower than in classical type 1 diabetes. The symptoms of metabolic syndrome are more seldom in LADA patients than in patients with type 2 diabetes. LADA patients account for about 10% of the phenotypically type 2 diabetic patients older than 35 years (Stenstrom et al. 2005; Tuomi 2005).

2.4.4 MODY

MODY (maturity-onset diabetes of the young) is a monogenic form of diabetes which manifests mostly in individuals with strong family history of diabetes. MODY typically starts at a young age (before age 25) and the patients do not require insulin therapy at the beginning, but later on insulin secretion is defective. The diabetic complications, including features of metabolic syndrome, are usually absent or mild. The severity of insulin secretion defect and other clinical symptoms varies in different MODY subtypes (Timsit et al. 2005). MODY3 seems to be the most common form of MODY in Scandinavia (Lehto et al. 1999). An estimation is that MODY accounts for less than 5% of all cases of diabetes (Velho et al. 1998).

2.5 Risk factors for diabetes

2.5.1 Genetic factors

Genetic susceptibility appears to be a prerequisite for type 1 diabetes and unknown environmental triggers determine who amongst susceptible people develop the disease (Daneman 2006). A strong familial aggregation is seen in the type 1 diabetes cases since about 10-13% of newly diagnosed children with type 1 diabetes have a first degree relative with the disease (Achenbach et al. 2005). The major determinants of the genetic predisposition to type 1 diabetes are the genes located in the HLA class II locus on chromosome 6 (Ilonen et al. 2002). The role of the molecules encoded by these genes is to present peptides derived from phagocytosed extracellular proteins to CD4+ (helper) T cells. The HLA class II molecules are expressed on the cell surfaces of specific antigen presenting cells, eg. B cells, dendritic cells and macrophages. Variants of HLA genes are thought to confer about 50% of the genetic susceptibility. The disease risk or probability is mainly defined by HLA DQ molecules. The high risk genotypes include DQA1*0301-DQB1*0302 (encoding protein HLA-DQ8), DQA1*0501-DQB1*0201 (encoding protein

HLA-DQ2) and DQA1*0301-DQB1*0201 (also encoding protein HLA-DQ2) (Ilonen et al. 2002). HLA-DR molecules and probably also other loci within the HLA region have a modifying effect on the HLA DQ defined genetic risk (She 1996). Thus the DQ8 haplotypes with HLA-DRB1*0403 or DRB1*0406 are associated with protection against the disease whereas DQ8 haplotypes with other DRB1*04 alleles are associated with disease risk. The protective HLA haplotypes include also (DR2)-DQA1*0102-DQB1*0602, (DR14)-DQA1*01-DQB1*0503 and (DR7)-DQA1*0201-DQB1*0303 (Ilonen et al. 2002). The individual risk is ultimately determined by the specific combination of risk and protection associated haplotypes.

Two other genes, CTLA-4 (cytotoxic T lymphocyte antigen-4) and insulin (INS) are thought to explain about 15% of the diabetes susceptibility (Daneman 2006). A VNTR (variable number of tandem repeats) and a series of associated SNPs (single nucleotide polymorphisms) in the insulin gene region have been associated with type 1 diabetes (Bennett et al. 1996). A variant of the gene PTPN22 (protein tyrosine phosphatase 22) (Bottini et al. 2004; Smyth et al. 2004), coding a suppressor of T cell activation, has been associated with type 1 diabetes as well. However, the HLA, CTLA-4 and PTPN22 genes have also been linked with other autoimmune diseases (Becker 1999; Bottini et al. 2004; Smyth et al. 2004), supporting the thought that overlapping biological pathways may contribute to different autoimmune diseases. In addition, more than ten other loci have been associated with type 1 diabetes (Concannon et al. 1998; Mein et al. 1998; Cox et al. 2001).

Type 1 and type 2 diabetes have been reported to have strong familial clustering: increasing frequency of type 2 diabetes has been detected in families with type 1 diabetes cases and type 1 diabetes cases in families with type 2 history. This suggests a common genetic susceptibility (Tuomi 2005). However, the genes associated with type 2 diabetes are still poorly known (Hansen et al. 2005).

LADA and MODY have been shown to have a genetic component as well. Patients with LADA appear to share the HLA conferred genetic susceptibility with type 1 diabetes since they have an increased frequency of HLA susceptibility alleles (Tuomi 2005). MODY is a dominantly inherited disease and mutations in six genes cause the majority of disease cases (Timsit et al. 2005).

2.5.2 Environmental factors

The genetic factors are not responsible alone for the development of type 1 diabetes and environmental triggers and accelerators are required for the process. The significant role of environmental factors is indicated by the rapid increase in diabetes incidence. It addition, not all of identical twins have type 1 diabetes (Barnett et al. 1981; Kaprio et al. 1992; Kyvik et al. 1995), also suggesting the crucial role of environmental factors in the disease development.

Various environmental and dietary factors have been suggested to be either risk factors or factors protecting against type 1 diabetes. The risk candidates include N-nitroso compounds (Helgason et al. 1981), cereals (Norris et al. 2003) and cow's milk. The effect of diet especially during infancy appears to be important, as it seems that early introduction of cow milk formula feeding could be diabetogenic and breastfeeding should be preferred (Vaarala et al. 1999; Virtanen et al. 2003; Sadauskaite-Kuehne et al. 2004). However, the results of the dietary and environmental factors on type 1 diabetes development are inconsistent and further studies are needed. In addition, virus infections during gestation and infancy, especially certain enterovirus, rotavirus and rubella infections, have been proposed to be associated with the development of type 1 diabetes (Ginsberg-Fellner et al. 1985; Hyoty et al. 1995; Horwitz et al. 1998; Honeyman et al. 2000). The factors suggested to protect against type 1 diabetes include nicotinamide, zinc, and vitamins C, D and E (Virtanen et al. 2003).

Environmental factors appear to be the main determinant of type 2 diabetes. Dietary factors, in particular high fat, high sugar, high salt and low fiber are central for the development of type 2 diabetes, in addition to other factors such as smoking and lack of exercise.

2.6 Humoral immunology of diabetes

2.6.1 History of autoantibodies

Bottazzo and colleagues were the first to discover by immunofluorescence that circulating autoantibodies against islets of Langerhans antigens exist in patients with type 1 diabetes (Bottazzo et al. 1974). Now it is known that these islet cell autoantibodies (ICAs) consist of many different antibodies against various intracellular islet

cell antigens. The next important finding was made in 1983 when Palmer and colleagues detected autoantibodies against insulin (IAA) from diabetic patients serum by a radioimmunoassay (Palmer et al. 1983). In 1990, Baekkeskov and colleagues identified by immunoblotting a pancreatic beta cell autoantigen sized 64 kDa to be glutamic acid decarboxylase (GAD), the biosynthesising enzyme of the neurotransmitter GABA (Baekkeskov et al. 1990). GAD is a molecule expressed in high levels in some CNS neurons (McLaughlin et al. 1975) and in pancreatic beta cells (Okada et al. 1976). Two isoforms of GAD (GAD65 and GAD67) have been identified (Karlsen et al. 1991; Michelsen et al. 1991) and both isoforms are expressed in the brain and the pancreatic islets. The 65 kDa isoform is the predominant form in the pancreas.

Christie and colleagues discovered in 1993 that type 1 diabetic patients have autoantigens sized 64 kDa which were distinct from GAD (Christie et al. 1993) and in 1994 it was shown that the islet cell antigen 512 (ICA512) is related to protein tyrosin phosphatases (Rabin et al. 1994). Finally in 1996, Lan and colleagues named this molecule as IA-2 (islet antigen 2) (Lan et al. 1996). Nowadays, these four autoantibody types (ICA, IAA, GAD65A, IA-2A) are tested from serum when predicting or diagnosing type 1 diabetes and they have gained a status as established markers of preclinical type 1 diabetes. ICA is tested by an indirect immunofluorescence method and IAA, GADA and IA-2A are tested by a radioimmunoprecipitation assays.

Recently, other autoantibodies in addition to the established ones have been searched for in patients with type 1 diabetes. Antibodies against antigens predominantly expressed in other tissues than pancreas, e.g. against vascular wall proteins such as elastin (Nicoloff et al. 2000) and basement membrane protein collagen type IV (Nicoloff et al. 2002), have been detected. Many of these antibodies against non-pancreatic tissues are present in human serum in several diseases, thus they are not useful in diabetes prediction or diagnosis. Antibodies to CD38 (ADP ribosyl cyclase/cyclic ADP-ribose hydrolase) have also been found from a minority of type 1 and type 2 diabetic patients but they either have not proved to be useful in predicting diabetes (Mallone et al. 2006).

2.6.2 Mechanism of the humoral autoimmunity

Type 1 diabetes is characterised by the appearance of diabetes-associated autoantibodies in the preclinical period, which likely reflects the ongoing beta cell damage. The series of events associated with the formation of autoantibodies against the pancreatic islet antigens are not well characterised. However, autoantibody production has been suggested to be a consequence of the T cell mediated beta cell destruction and the following processing of various intacellular beta cell autoantigens. This could result in B lymphocyte activation and autoantibody production (Falorni et al. 2003). It is not known why the normal suppression of autoreactivity fails in patients with type 1 diabetes. It is either not known whether autoantibodies are critically involved in the initiation or progression of the disease (Roep 2003).

Epitope spreading is the mechanism by which autoantibodies against multiple antigens are thought to develop in type 1 diabetes. It is a process whereby the initial immune response against one inducing epitope is extended to other epitopes distinct from and non-cross-reactive with the original epitope. Thus a wide variety of epitopes can become targets of the ongoing immune response. Spreading of autoimmunity is observed within one molecule but also between different molecules (Achenbach et al. 2005).

2.6.3 Appearance and prevalence of autoantibodies

The latent preclinical phase of type 1 diabetes may last for several years but in some cases the disease progresses into clinical diabetes within a few months; it appears to be that younger individuals with the autoantibodies progress faster to the clinical disease than older persons (Daneman 2006). At the time of diagnosis, almost all subjects with diabetes are autoantibody positive (Sabbah et al. 1999).

The genetically susceptible children who develop diabetes-associated autoantibodies early in infancy typically develop type 1 diabetes in early childhood. They often develop autoantibodies already before the age of 2 years. These children most often develop multiple islet autoantibodies and the prevalence of autoantibody positivity increases with age (Ziegler et al. 1999; Kimpimaki et al. 2001; Hummel et al. 2004). The order of diabetes-associated

autoantibody appearance usually is first IAA, then GADA and IA-2A in these individuals (Achenbach et al. 2005). On the contrary, children who develop the first autoantibodies later in their life have slower progression to multiple autoantibodies and to type 1 diabetes (Hummel et al. 2004). Specific HLA genotypes appear to have an influence on the timing of appearance of autoantibodies and the magnitude and extent of the autoimmune response (Schenker et al. 1999; Yu et al. 2000; Knip et al. 2002; Hermann et al. 2005).

The persistence of diabetes-associated antibodies from the time of diabetes diagnosis to ten years after diagnosis was studied by Savola et al (Savola et al. 1998b). The concentrations and prevalences of IA-2A and ICA decreased considerably during the course of the disease, but still after 10 years 67% of the subjects had at least one autoantibody detectable. GADA levels showed substantial fluctuation during the ten year follow-up period. Generally, the prevalence of the diabetes-associated autoantibodies has a tendency to decrease after the diagnosis of the disease.

The current view is that the more antibody types detected, the greater the possibility to develop clinical diabetes, e.g. subjects who have positivity for 3-4 antibodies have a 60-100% risk of developing type 1 diabetes over the next 5-10 years (Verge et al. 1996; Gardner et al. 1999; LaGasse et al. 2002). However, many who have signs of beta cell autoimmunity do not progress to the clinical disease (Knip 2002). Recent reports have suggested that a combination of autoantibody screening and HLA genotype screening (Kupila et al. 2001; Decochez et al. 2005), the deciphering of the distribution of various immunoglobulin G (IgG) subclasses and reactivities against specific epitopes (Achenbach et al. 2004) or the IA-2A screening alone (Decochez et al. 2002; Knip et al. 2002; Decochez et al. 2005) would be more appropriate markers of developing diabetes. It has also been suggested that GADA would be rather markers of general autoimmunity than type 1 diabetes (Knip et al. 2002). The autoantibody profile varies with the age at onset and sex; in subjects of different ages, different autoantibodies are regarded as more sensitive predictors of the development of diabetes (Pihoker et al. 2005).

In addition to patients with classical type 1 diabetes, the diabetes-associated autoantibodies are detected in

LADA patients. Typically LADA patients have GAD antibodies but also other islet autoantibodies are detected. In LADA patients similarly to type 1 diabetes patients the number of autoantibody types detected seems to correlate with the speed of beta cell loss; the more antibody types detected the faster the beta cell deterioration (Stenstrom et al. 2005).

2.7 Diabetic nephropathy

Diabetic nephropathy (DN) is a severe complication of diabetes affecting approximately 25-40% of both type 1 and type 2 diabetic patients. Poor glycemic control seems to be a prerequisite for the kidney disease and the first signs of DN generally appear within 5-15 years of diabetes duration. DN is the single most common disease leading to renal failure in adults and a major cause for high mortality rates in diabetic patients (Gross et al. 2005).

The onset of DN is associated with an increased glomerular filtration rate (GFR). The subsequent stages present with elevated blood pressure and damage of the kidney glomerular filtration barrier resulting in the loss of large blood proteins, such as albumin into the urine (proteinuria). This is followed by progressive scarring of the kidney tissue and, ultimately, end-stage renal disease with decline in GFR and total loss of kidney function. The series of structural changes detected in the kidney glomerulus include thickening of the glomerular basement membrane, glomerular mesangial cell growth, mesangial matrix expansion, mesangial sclerosis (Mogensen 1976) and podocyte loss (Pagtalunan et al. 1997; White et al. 2002). In addition, the expression of the key molecule of the slit diaphragm, nephrin, is altered in DN (Doublier et al. 2003).

The risk factors for DN in addition to hyperglycemia include increased blood pressure levels, smoking, elevated serum lipids and dietary factors such as the amount and source of protein (Gross et al. 2005). Genetic predisposition has also been suggested as a risk factor (Rich 2006; Sale et al. 2006) but, thus far, none of the DN associated genes have been shown to have as significant a role in the disease process as HLA genes have in type 1 diabetes.

The mechanisms by which the increased intra- and extracellular glucose concentration in diabetic patients could lead to kidney damage have been

suggested to include increased oxidative stress (Giugliano et al. 1996), advanced glycation end-product formation, activation of various signaling pathways, increased polyol and hexosamine pathway flux (Brownlee 2001) and downregulation of nephrin expression in the podocytes (Doublier et al. 2003; Menne et al. 2006). In addition, inflammation, including infiltration of lymphocytes into the kidney tissues and production of cytokines, chemokines and growth factors, has been implicated with the development of DN (Bohle et al. 1991; Furuta et al. 1993; Saraheimo et al. 2003; Navarro et al. 2005).

Diabetic nephropathy is categorised in stages on the basis of the urinary albumin excretion rate (AER): microalbuminuria as AER between 30-300 mg/24h or 20-200 µg/min and macroalbuminuria/proteinuria as AER >300 mg/24h or >200 µg/min. Microalbuminuria is used as a marker for DN (Mogensen et al. 1984). Recently, Caramori and colleagues suggested that looking at the combination of patient parameters (eg. AER, family history, blood pressure, plasma lipid levels) instead of the AER alone would be more accurate in the assessment of the DN risk (Caramori et al. 2006). Strict glycemic and blood pressure control have been proven successful in slowing down the clinical and structural manifestations of DN in type 1 diabetic patients in many studies, but despite these advancements the cases of diabetic kidney disease are still increasing (Caramori et al. 2006).

2.7.1 Autoimmunity in diabetic nephropathy

Already in the 1970s, Mauer and colleagues found linear deposits of immunoglobulin and complement in the glomeruli of diabetic rats (Mauer et al. 1972). Since then autoimmunity has been suggested as one of the mechanisms participating in the development of DN. Interestingly, in the CNF patients who have undergone a renal transplant, the recurrence of severe proteinuria has been suggested to be caused at least partially by nephrin antibodies (Wang et al. 2001; Patrakka et al. 2002). This implies that the antigens of the podocyte cells in the kidney glomerulus are accessible to circulating antibodies.

There are few studies which have considered the association of specific autoantibodies with DN. However, no association

has been detected between the established diabetes-associated autoantibodies, GADA and ICA, and diabetic complications (Roll et al. 1995; Vinik et al. 1995), suggesting that these autoantibodies have no pathogenic role in longlasting diabetes and DN. Autoantibodies against the glycosphingolipid sulphatide, a molecule expressed in the neurons (Fredman et al. 1991), in the pancreatic Langerhans islets and in the kidneys (Buschard et al. 1993), have been detected in patients with type 1 diabetes (Buschard et al. 1993) but the data did not suggest an association of these antibodies to diabetic complications (Andersson et al. 2002). The prevalence and significance of phospholipid autoantibodies in the circulation of diabetic patients has also been studied, but they did not appear to be associated with DN (Vinik et al. 1995).

Oxidised and glycated proteins are commonly found in diabetic patients as a results of increased hyperglycemia and oxidative stress (Giugliano et al. 1996). Oxidised LDL is also known to be immunogenic. Autoantibodies against glycated and oxidased LDL in diabetic patients have not been shown to associate with DN (Korpinen et al. 1997; Leinonen et al. 1998), but interestingly, high concentrations of oxidised LDL-anti-oxidised LDL complexes were associated with proteinuria (Atchley et al. 2002). These results suggest that hyperglycemia and hyperlipidemia seen in diabetic patients can induce the formation of immune complexes, which could play a role in the pathogenesis of diabetes-related vascular complications; the immune complexes could be deposited in the small blood vessels of microcirculation, eg. in small glomerular vessels. This view was strengthened by the finding of Nicoloff and colleagues who showed that circulating immune complexes associate with the development of microvascular complications in diabetic children (Nicoloff et al. 2004). Albeit the many studies considering autoantibodies in diabetic nephropathy, none of the reports have been able to demonstrate that specific antibodies would have an active role in the development of the diabetic kidney disease.

3 Aims of the study

The aim of this thesis work was to systematically study the expression of nephrin in human lymphoid cells and tissues, and furthermore, to decipher if there are podocyte proteins in the pancreas with similarities with the complex in the slit diaphragm area. In addition, I explored the possibility whether nephrin, densin and filtrin can act as autoantigens and if autoantibodies against these molecules can be detected in the serum of patients with type 1 diabetes. The possible association of these autoantibodies to diabetic nephropathy was also deciphered.

The specific aims of this thesis work were:

- To find out whether other slit diaphragm-associated proteins than nephrin can also be found in pancreatic islet cells

- To determine the expression of nephrin in lymphoid cells and tissues

- To identify nephrin autoantibodies (NAA) in serum samples of patients with type 1 diabetes

- To identify densin and filtrin autoantibodies (DAA and FAA) in serum samples of patients with type 1 diabetes

- To study the association of NAA, DAA and FAA with developing diabetic nephropathy

4 Subjects, materials and methods

4.1 Human study subjects

4.1.1 University of Oulu patient material (Publication III, IV)

The autoantibody study population comprised of children and adolescents (Table 3) diagnosed with type 1 diabetes at the Department of Paediatrics, University of Oulu, Finland between 1983 and 1986 (Savola et al. 1998b). The total follow-up period for the detection of diabetic complications was 17-19 years. In addition to the patient serum samples, samples of the Finnish healthy control population (Table 3), gathered between the years 1989-1994, were measured, and the results were used to define the cut-off limits for autoantibody positivities. All samples were stored at –20°C until analysed.

Detailed clinical data of the samples as well as titres of four established diabetes-associated autoantibodies, i.e. ICA, GADA, IAA and IA-2A were available (Savola et al. 1998a). Normoalbuminuria was defined as albumin excretion rate of less than 30mg/24h, microalbuminuria as 30-300mg/24h and proteinuria/ macroalbuminuria as more than 300mg/24h. During the time of this study, 10 patients dropped out from the follow up. Of these patients nine had moved to another region in Finland and one had died from multiple sclerosis. The mean duration of diabetes when patients dropped out from the study was 8.7 years (range 4 to 13 years).

	Number of subjects	Gender Male/female	Mean age at diagnosis in years	Serum sample timepoints (years from diagnosis)
NAA study				
Patients	66	43/23	8.2	0, 2, 5, 10
Healthy subjects	96	48/48	11.3	0
DAA study				
Patients	66	43/23	8.2	0, 2, 5, 10
Healthy subjects	90	43/47	11.4	0
FAA study				
Patients	66	43/23	8.2	0, 2, 5, 10
Healthy subjects	77	39/38	11.1	0

***Table 3**. University of Oulu study material utilised in the nephrin, densin and filtrin autoantibody studies. The gender distribution and mean age of the subjects are indicated. There was a variation in the number of healthy subjects in each assay. NAA, nephrin autoanti-bodies; DAA, densin autoantibodies; FAA, filtrin autoantibodies.*

4.1.2 FinnDiane patient material (unpublished)

Serum samples from a subset (n=150) of type 1 diabetic patients from an ongoing nationwide, multicentre Finnish Diabetic Nephropathy Study (FinnDiane) were utilised in this study (Saraheimo et al. 2003). The patients were divided into three groups according to their AER from three consecutive overnight or 24-h urine collections: normoalbuminuric (n=50), microalbuminuric (n=50) and macroalbuminuric (n=50). Type 1 diabetes was defined as an onset of diabetes before the age of 35 years and permanent insulin treatment initiated within one year of diagnosis. The duration of diabetes required was at least ten years. The mean age of the patients was 36.0 years, mean age of onset of diabetes was 13.4 years and the mean diabetes duration was 22.6 years. Patients with normal AER had no antihypertensive medication but patients with microalbuminuria and macroalbuminuria had angiotensin converting enzyme inhibitor treatment. Thirty five healthy control subjects were included in the study. Detailed clinical data were available including clinical history, data on medication, cardiovascular status, diabetic complications, total glycated hemoglobin and creatinine (Saraheimo et al. 2003).

4.2 Tissues and cells

4.2.1 Tissue samples (I, II, III, IV)

Human pancreas, kidney, thymus, lymph nodes, tonsil, adenoid tissues, rat pancreas and kidney tissues and mouse pancreas tissues were used in these studies. Normal rat pancreas, rat kidney and mouse pancreas tissues were obtained from our previous experiments. Human pancreas and kidney tissues were obtained from human cadaver donors from Helsinki University Central Hospital. Human thymic tissue was obtained during open-heart surgery at the Hospital for Children and Adolescents, Helsinki University Central Hospital. Lymph node tissues were gathered from the routine diagnostic biopsy material of the Central Laboratory of Pathology, Helsinki University Central Hospital. Human tonsil and adenoid tissues were kindly provided by Dr. P. Mattila (Department of Otorhinolaryngology, Helsinki University Central Hospital).

4.2.2 Isolation of cells from human peripheral blood (II)

Human peripheral blood mononuclear cells were isolated from buffy coat preparations by Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) gradient separation. The monocyte population was enriched by two hours adherence to culture plates. The adherent cells were incubated in RPMI 1640 media (BioWhittaker, Verviers, Belgium) supplemented with 10% foetal calf serum (Life Technologies, GibcoBRL, Karlsruhe, Germany), 100 U/ml penicillin/streptomycin, two mM glutamine in humidified atmosphere with 5% CO2 at 37°C. Incubation of adherent cells in Macrophage-SFM medium (Life Technologies, Gaithesburg, MD) for seven days in the presence of 600 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF) gave rise to macrophages (Pirhonen et al. 2001), whereas presence of 500 U/ml GM-CSF (Roche Diagnostics GmbH, Mannheim, Germany) and 250 U/ml interleukin-4 (IL-4) (Roche Diagnostics) for seven days gave rise to dendritic cells (Sallusto et al. 1994). By a further incubation with added lipopolysaccharide (50 ng/ml) or tumour necrosis factor- (TNF- , 10 ng/ml) for 48 hours, the dendritic cells were matured (Sallusto et al. 1994) and the macrophages were activated (Rodenburg et al. 1998).

Various lymphocyte populations were isolated from peripheral blood mononuclear cells using a magnetic cell separation system,

(Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). For the isolation of T lymphocytes, monocytes and B lymphocytes fluorescein isothiocyanate (FITC)-conjugated antibodies against, CD3 (Pharmingen, San Diego, CA), CD14 (Dako, Glostrup, Denmark) and CD19 (Becton Dickinson, San Jose, CA) were used respectively together with magnetic beads coated with anti-FITC or anti-mouse Ig antibodies. The purity of the isolated cell populations was over 90% as verified by FACS analysis.

4.3 Messenger RNA (mRNA) expression

4.3.1 Database searches (I)

The expressed sequence tag (EST) libraries at the National Center for Biotechnology Information (NCBI) Unigene database (http://www.ncbi.nlm.nih.gov/) (Wheeler et al. 2003) were screened to assess the potential expression of the selected podocyte molecules at the mRNA level in pancreatic tissue and in pancreatic cell lines.

4.3.2 RNA extraction and synthesis of complementary DNA (cDNA) (I, II, IV)

Total RNA was isolated with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) from a variety of tissues and cells: human pancreas, kidney, thymus, adenoid and tonsil, cultured dendritic cells and macrophages and magnetically isolated peripheral blood cells. The RNA was treated with DNase I (Promega, Madison, WI, USA) and transcribed into cDNA using poly-T priming (Roche, Basel, Switzerland) and Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (reverse transcriptase positive, RT+ sample, Promega). To confirm the RNA origin of the reverse transcriptase-polymerase chain reaction (RT-PCR) signals, samples that were not reverse transcribed (RT-) were analysed in parallel with the RT+ samples.

4.3.3 Reverse transcriptase-polymerase chain reaction (RT-PCR) (I, II, III, IV)

The primers for RT-PCR were designed with the Primer3 software (Rozen et al. 2000), and their specificity was verified by using the Blast search algorithm in the Genbank database at the NCBI (Altschul et al. 1990; Benson et al. 2000). The primers are summarised in Table 4.

The RT-PCR reactions were performed using EXT DNA polymerase (Publication I, IV) (Finnzymes, Espoo, Finland) or AmpliTaq Gold DNA polymerase (Publication II, III) (Perkin Elmer, Norwalk, CT, USA) with initial denaturation of cDNA at 95 °C for 3 min, followed by 35-40 amplification cycles (95 °C 30 s, 56-58 °C 1 min, and 72 °C 30 s), and final elongation at 72 °C for 5 min. The reactions were run in agarose gels, the RT-PCR products were purified and sequenced as described earlier (Palmen et al. 2001).

Target (Genbank number)	Primer sequence	Used in	Study
CD2AP (AF164377)	5′-ccaaagatgcctggaagaag-3′	RT-PCR	I
	5′-agaagcactggaaggtgttga-3′	RT-PCR	I
Podocin (AJ279254)	5′-cgcacaaggagaacaagagg-3′	RT-PCR	I
	5′-gagtttggagacgaaggtcaa-3′	RT-PCT	I
	5′-caaagtgcggatgattgct-3′	RT-PCR	I
	5′-aggatttagtggctcaacagg-3′	RT-PCR	I
	5′-atcgcccaagatgcaaag-3′ *	RT-PCR	I
	5′-tgaagggtgtggaggtatcg-3′ *	RT-PCT	I
NEPH1 (AY017369)	5′-ccagaggacaccaggattga-3′	RT-PCR	I
	5′-ttcggcaagtgaagacacg-3′	RT-PCR	I
NEPH2 (AF480410)	5′-catcatctccagcacccaga-3′	RT-PCR	I
	5′-tgccataaggacgaggaagg-3′	RT-PCT	I
	5′-ccggagtggtcctgagcaat-3′	RT-PCR	I
	5′-cttgatctggcccttctcgc-3′	RT-PCR	I
Filtrin (NM_199180)	5′-tgaccatctgcattgagagg-3′	RT-PCR	I
	5′-tccttgggtccaagtctttc-3′	RT-PCT	I
FAT (NM_005245)	5′-cctctgaacctcacccacat-3′	RT-PCR	I
	5′-ccacagacacctttтcatcg-3′	RT-PCR	I

Synaptopodin	5′-attgacatccagcccaacac-3′	RT-PCR	I
(NM_007286)	5′-catcatcctcctttccacca-3′	RT-PCT	I
	5′-tgccaaggtctcaccaaga-3′	RT-PCR	I
	5′-ggctgctcatgctgctc-3′	RT-PCR	I
Alpha-actinin-4	5′-tgcagaacttccacatcagc-3′	RT-PCR	I
(NM_004924)	5′-aaggcatggtagaagctgga-3′	RT-PCT	I
Nephrin (AF035835)	5′-cccatcactacccccggtct-3′	RT-PCR	I, II
	5′-gagacaacacgactggcac-3′	RT-PCT	I, II
Beta-actin (BC016045)	5′-aaccgcgagaagatgacccagatcatgttt-3′	RT-PCR	II
	5′-agcagccgtggccatctcttgctcgaagtc-3′	RT-PCR	II
Nephrin (AF035835)	5′-caactgggagagactgggagaa-3′ (primer)	Q RT-PCR	II
	5′-aatctgacaacaagacggagca-3′ (primer)	Q RT-PCR	II
	5′-tccacaatgcactggtaagcgcca-3′ (probe)	Q RT-PCR	II
UbcH5B (U39317)	5′-tgaagagaatccacaaggaattga-3′ (primer)	Q RT-PCR	II
	5′-caacaggacctgctgaacactg-3′ (primer)	Q RT-PCR	II
	5′-tgatctggcacgggaccctcca-3′ (probe)	Q RT-PCR	II
Nephrin (AF035835)	5′-tcgaagcttgccgccaccatggtcaactgggagagactg-3′	Antigen generation	III
	5′-tacgtagaattcttacaccagatgtcccctcagctcgaa-3′	Antigen generation	III
Densin (AF434715)	5′-gtcccagcaaagcaacatt-3′	RT-PCR	IV
	5′-tccaccactgatactaaatccaag-3′	RT-PCR	IV
	5′-ctggtaccaccatggacaagccatcagataaca-3′	Antigen generation	IV
	5′-agagctcttacctttgccctttagtttgaat-3′	Antigen generation	IV
Filtrin (NM_199180)	5′-gctctagaaccatggcatggcgccacagcaaggc-3′	Antigen generation	IV
	5′-gcgagctctcacacgtgagtctggaga-3′	Antigen generation	IV

Table 4*. Primers and probes used in conventional RT-PCR (RT-PCR), Real-time quantitative RT-PCR (Q RT-PCR) and in antigen generation. The podocin primers which amplified the podocin sequence from the pancreas are marked with an asterisk (*).*

4.3.4 Real-time quantitative RT-PCR (II)

For the amplification of human nephrin with the Taqman Real-time quantitative RT-PCR (Q RT-PCR), specific primers and FAM (6-carboxy-fluorescein)-labelled probe were used (Table 4). The ubiquitin conjugating enzyme (UbcH5B) gene, used as the endogenous control, was amplified using the sense and antisense primer pair as well as the VIC-labelled probe (Hamalainen et al. 2001) (Table 4). The UbcH5B values were used to normalise the amounts of nephrin. The ABI Prism 7700 sequence detector, which was used for the signal detection, was programmed to an initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 thermal cycles of 15 s at 95°C and 1 min at 60°C.

4.4 Protein expression

4.4.1 Tissue and cell lysates and immunoblotting (I, II)

Human glomeruli were prepared as described by Kerjaschki et al (Kerjaschki et al. 1983). Rat kidney and pancreas tissue, human kidney, pancreas, thymus, adenoid and tonsil tissues were homogenised in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl (pH 8)), urea buffer (8 M urea, 1 mM dithiothreitol (DTT), 1 mM EDTA) or Laemmli buffer (94 mM Tris-HCl (pH 6.8), 13% glycerol, 3% SDS, 7.5% 2-mercaptoethanol, 0.075% bromophenolblue). Cell extracts were centrifuged at 10 000g for 10-30 min at 4°C. Proteins from the magnetically separated cell populations, the monocyte-derived dendritic cells and the monocyte-derived macrophages were extracted from the phenol phase, obtained when isolating RNA with Trizol® (Life Technologies) reagent. Protein concentrations in protein extracts were determined using BCA Protein Assay reagent (Pierce, Rockford, IL, USA).

Appropriate amounts of the cell or tissue lysates were run through reducing polyacrylamide gels in the Protean Mini-gel electrophoresis system (Bio-Rad Laboratories, Richmond, CA, USA) and the proteins were transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) using a wet-transfer system. After blocking in 2-5% skimmed milk (Valio, Finland), the membranes were incubated with the primary antibodies (Table 5) for at least one hour followed by incubation with appropriate horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and detection of the signals with SuperSignal ECL substrate (Pierce, Fockford, IL, USA).

Primary antibody	Poly/monoclonal	Source/reference	Used in
CD2AP	P	Santa Cruz, (Shih et al. 2001)	I
Podocin	P	(Roselli et al. 2002)	I
FAT	P	(Inoue et al. 2001)	I
NEPH1	P	(Liu et al. 2003; Sellin et al. 2003)	I
Filtrin	P	(Ihalmo et al. 2003)	I
Synaptopodin	M	Progen, (Mundel et al. 1997)	I
Alpha-actinin-4	M	(Honda et al. 1998; Kaplan et al. 2000)	I
Nephrin	P	(Ahola et al. 2003)	II

Table 5*. Primary antibodies used in immunoblotting experiments. P, polyclonal antibody; M, monoclonal antibody.*

4.4.2 Immunostaining of tissue sections

4.4.2.1 Immunofluorescence (I, II, III, IV)

Frozen sections (4-6 µm) of human, rat or mouse pancreas, human adenoids and rat kidney were fixed with acetone or 3.5-4% paraformaldehyde for two to ten min, washed with phosphate-buffered saline (PBS), blocked with 10% normal goat serum or CAS-Block (Zymed Laboratories, San Francisco, CA, USA), and incubated with the primary antibodies (Table 6) for one hour or overnight (o/n). After washes with PBS, the sections were further incubated for one hour with appropriate fluorescent secondary antibodies. After washes, the sections were embedded in Immu-Mount embedding medium (Shandon, Pittsburgh, PA, USA) or Vectashield mounting medium for immunofluorescence (Vector Laboratories, Inc., Burlingame, CA, USA). The control stainings, by omitting the primary antibodies and by pre-immune sera, were performed when possible. The sections were examined with a Zeiss Axiophot2 microscope (Carl Zeiss Jena GmbH, Jena, Germany) or Olympus Provis microscope (Olympus America Inc, FL, USA).

Antibody/serum	Poly/monoclonal	Reference/Source	Used in
CD2AP	P	Santa Cruz Biotechnology (CA,USA)	I
Podocin	P	(Roselli et al. 2002)	I
FAT	P	(Inoue et al. 2001)	I
NEPH1	P	(Sellin et al. 2003)	I
Filtrin	P	Ihalmo et al, submitted	I
Synaptopodin	M	Progen (Heidelberg, Germany)	I
Alpha-actinin-4	M	(Honda et al. 1998)	I
Nephrin (anti-rat)	P	(Ahola et al. 2003)	I, III
Insulin	M	Neomarkers (Fremont, CA, USA)	I, IV
Nephrin (anti-human)	P	(Ahola et al. 2003)	II
CD3	P	Becton Dickinson (San Jose, CA, USA)	II
CD19	P	Dako (Glostrup, Denmark)	II
CD21	M	Dako (Glostrup, Denmark)	II
Human sera	Not determined	(Savola et al. 1998b)	III
Densin	P	(Izawa et al. 2002)	IV

Table 6. *Primary antibodies used in immunofluorescence experiments. P, polyclonal antibody; M, monoclonal antibody.*

4.4.2.2 Immunohistochemistry (II)

Lymph node sections were deparaffinised in xylene and rehydrated through graded concentrations of ethanol to distilled water. The slides were incubated in a microwave owen in 10 mM Tris-HCl, 1mM EDTA buffer, pH 9.0 for 20 min. The immunohistochemical staining was performed using a Lab Vision Autostainer (Lab Vision Corporation, Fremont, CA, USA) and ChemMate DAKO Envision Detection Kit (DakoCytomation, Glostrup, Denmark). The sections were incubated with a primary antibody against nephrin (diluted 1:500, (Ahola et al. 2003)) for 30 min. Rabbit IgG and antibody dilution buffer were used instead of a primary antibody as negative controls. The staining was visualised using ChemMate DAB chromogen (DakoCytomation) and the sections were counterstained in Mayer's hematoxylin (Merck, Darmstadt, Germany).

4.5 Autoantibody studies by radioimmunoprecipitation assay (RIA)

4.5.1 Generation of antigens for RIA (III, IV, unpublished)

The antigens used in the RIA were developed by PCR, subcloning and in vitro transcription-translation. The primers used to produce recombinant intracellular parts of human densin, filtrin and nephrin are described in Table 4. The inserts were cloned into a pGEM-4z circular plasmid vector (Promega) under T7 promoter sequence using specific restriction sites generated to the ends of the inserts. The plasmids were transformed into Escherichia coli Top-10 DH5- (Invitrogen) cells using standard techniques. The purification of the plasmids for in vitro transcription-translation (IVT) was performed with a plasmid purification kit (HiSpeed Plasmid Maxi Kit, Qiagen). To verify that the cloning was successful and no mutations were present, PCR and sequencing were performed.

In vitro transcription-translation with a TnT Coupled Reticulocyte Lysate System (Promega) and Redivue L-[35S]-methionine (Amersham Biosciences, Buckinghamshire, UK) was used to produce the 35S-methionine labeled recombinant proteins from the purified plasmids. Unincorporated 35S-methionine was removed by gel chromatography on a NAP-5 column (Amersham Biosciences, Uppsala, Sweden). The size of the translated proteins were characterised by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography of the dried gel on film overnight.

4.5.2 RIA (III, IV, unpublished)

The labelled recombinant proteins produced by in vitro transcription-translation were used as antigens in the radioimmunoprecipitation assay. Two or four µl of serum samples were incubated with 9000-15 000 counts per minute (cpm) of the 35S-labeled densin, filtrin or nephrin recombinant protein in a total volume of 50 µl in TBST (Tris-buffered saline with Tween-20, 50 mmol/l Tris, 150 mmol/l NaCl (pH 7.4), 0.1% Tween 20) for 7-18 hours. To isolate the immune complexes 8 µl of protein A-sepharose 4B conjugate (Zymed Laboratories, San Francisco, California, USA) was added to a total volume of 50 µl of TBST. After incubation for one to two hours the samples were washed eight times with 140 µl of TBST. Scintillation cocktail (Optiphase 'SuperMix' or Optiphase 'HiSafe' 3, Wallac, Turku, Finland) was added and the activity measured with a liquid scintillation counter (1450 MicroBeta Trilux; Wallac). All incubations were performed at 6°C on a shaker and each serum sample was measured at least as a duplicate.

4.5.3 Densin, filtrin and nephrin autoantibody measurements (III, IV, unpublished)

For each of the antigen-specific RIAs, a dilution series of a serum pool or an antibody against a specific molecule were utilised in standardisation: a polyclonal antibody against densin (Apperson et al. 1996), a polyclonal antibody against nephrin (Ahola et al. 2003) and a serum pool with high titre of FAA. These dilutions were given relative values of 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125, respectively. The standard curve of the dilution series on each plate was used to diminish the effect of interassay variation and to give the actual test samples a value in relative units (RU). The intra- and interassay coefficients of variation, mean levels of autoantibodies in healthy controls and cut-off limits for autoantibody positivity (mean+2.09 standard deviation, SD) for the University of Oulu patient material are presented in Table 7.

	Intra-assay coefficient of variation	Interassay coefficient of variation	Mean level in healthy controls in RU (±SD)	Cut-off limit for positivity, RU
NAA study (III)	4.7	17.2	0.066 (0.015)	0.1
DAA study (IV)	4.6	24.9	0.1 (0.05)	0.196
FAA study (IV)	5.3	3.9	0.018 (0.06)	0.143

Table 7. *The main variables in the autoantibody studies. The cut-off limit for autoantibody positivity was determined by utilising mean autoantibody relative units (RU) in the healthy controls (mean +2.09 SD). NAA, nephrin autoantibodies; DAA, densin autoantibodies; FAA, filtrin autoantibodies.*

4.5.4 Statistical analysis (III, IV)

Statistical analysis software (SPSS 12.0.1 for Windows; SPSS, Chicaco, IL, USA) was used to maintain a database of the patient and healthy control sample data, produce descriptive statistics of the groups and to test the correlation between the autoantibody levels (DAA, FAA, NAA) and other patient data parameters. Categorical variables were compared with chi-square statistics. Normally distributed continuous variables were tested with a two-tailed t-test for independent samples and analysis of variance. Nonparametric Mann-Whitney U-test and Wilcoxon-signed Rank test were applied to compare skewly distributed variables. Kaplan-Mayer survival curve was constructed for the time of diagnosis of diabetes to the time of diagnosis of microalbuminuria or proteinuria (III). Spearman's non-parametric correlation analysis and Pearson correlation test were used to assess correlations. P-values <0.05 were considered significant.

4.6 Ethical issues (I, II, III, IV, unpublished)

The studies utilising human serum and tissue samples (Publications I, II, III, IV, unpublished) followed the principles of the Declaration of Helsinki. The study performed with the University of Oulu patient material was approved by the Ethics committee of the University of Oulu, and

the FinnDiane study protocol was approved by the local Ethics committee in each participating study centre. Written informed consent was obtained from each subject participating in the studies III, IV. The use of human tissues was approved by the Ethics Committee of the Helsinki University Central Hospital. The use of animal tissues (Publications I, III) was approved by the Ethics Committee of the University of Helsinki.

5 Results

5.1 Podocyte molecules in the pancreas (I, IV)

5.1.1 Pancreatic EST libraries (I)

To find out which of the selected podocyte molecules (CD2AP, NEPH1, NEPH2, filtrin, FAT, synaptopodin, alpha-actinin-4, podocin and densin) are expressed in the pancreas at the mRNA level, screening of pancreatic expressed sequence tag (EST) libraries was performed. The ESTs of all the studied molecules, except for podocin and densin, were found in the pancreatic expression libraries according to the Unigene database.

5.1.2 Expression at gene and protein level (I, IV)

The expression of the selected podocyte molecules was studied at the mRNA level in the human pancreas (primers in Table 4). The mRNA expression of nephrin, CD2AP, FAT, alpha-actinin-4, NEPH1, filtrin, synaptopodin and densin were clearly detected. Only one podocin primer pair out of three (790-1052 base pairs, Table 4) showed podocin-specific PCR signal in the pancreatic cDNA. NEPH2 mRNA expression could not be detected in the human pancreas.

The expression of the podocyte molecules in the pancreas at the protein level was studied by immunoblotting. Protein bands of comparable size in the pancreas and the kidney lysates were detected for CD2AP, filtrin, alpha-actinin-4 and FAT. The podocin antibody recognised the major immunoreactive band of ~35 kDa in the pancreas lysate instead of the band at ~44 kDa in kidney glomeruli. The presence of synaptopodin and NEPH1 in the pancreas could not be detected.

5.1.3 Localisation (I, IV)

To elucidate the localisation of the podocyte proteins in the pancreas, pancreatic tissue sections were double-stained with antibodies to selected proteins and insulin or nephrin. Nephrin and insulin partially colocalised in the Langerhans islets as shown by an antibody against the whole intracellular part of nephrin. However, some staining with the antibody could be detected in other islet cell types as well. FAT,

filtrin, alpha-actinin-4 and densin positive staining was also found in the islets of Langerhans, mostly in the insulin producing beta cells. However, the colocalisation of FAT, filtrin and densin with insulin did not appear exclusive, suggesting that these proteins may also be expressed in other Langerhans islet cells than the beta cells. In addition, FAT and alpha-actinin-4 antibodies showed some staining in the exocrine pancreas. Stainings with synaptopodin and NEPH1 antibodies did not show a clear specific signal, which is consistent with the finding that neither of these proteins could be detected in the pancreas immunoblot. Podocin staining was not detected in the Langerhans islet cells but rather in the exocrine part of the pancreatic tissue. No staining with the CD2AP antibody was detected in the pancreas leaving the localisation of CD2AP undefined. As a summary, nephrin, FAT, filtrin, alpha-actinin-4 and densin expression was found in the Langerhans islet beta cells.

5.2 Nephrin in human lymphoid tissues (II)

5.2.1 Gene expression

Nephrin mRNA expression in the lymphoid tissues and cells was studied with RT-PCR and Real-time quantitative RT-PCR. Nephrin cDNA was detected in the human thymus, adenoid and tonsil tissues. In addition, nephrin was detected in the T lymphocytes (CD3+), monocytes (CD14+) and B lymphocytes (CD19+) isolated from human peripheral blood and monocyte-derived dendritic cells and macrophages after TNF- stimulation. The RT-PCR products from lymphoid tissues and cells were identical to the nephrin sequence in the kidney. The monocyte-derived macrophages, produced in the presence of GM-CSF after seven days of in vitro culture, showed no nephrin expression by conventional RT-PCR.

Real-time quantitative RT-PCR was used to quantitate nephrin mRNA levels. Nephrin expression was about 34% in the tonsil, 26% in the adenoid and 6% in the thymus of the expression level detected in the kidney. The T lymphocytes, B lymphocytes, monocytes and monocyte-derived dendritic cells and macrophages showed negligible nephrin mRNA expression and no nephrin was detected in the monocyte-derived macrophages.

5.2.2 Protein expression

Nephrin protein expression in the lymphoid tissues and cells was studied by immunoblotting. The nephrin antibody recognised a 185 kDa and a smaller 165 kDa protein band from kidney cortex lysate as well as a protein of 165 kDa in human tonsil and adenoid lysates. The signal from the adenoid and tonsil could be blocked with pre-incubation of the antibody with the respective antigen used in immunisations, verifying specificity of the antibody. No protein band with the nephrin antibody was detected in the immunoblot from the monocyte-derived dendritic cells or monocyte-derived macrophages or in the lysates from human T lymphocytes (CD3+), monocytes (CD14+) or B lymphocytes (CD19+) separated from the peripheral blood.

5.2.3 Localisation

Localisation of nephrin in the lymphoid tissues was studied by immunohistochemical staining. Staining of lymph node sections with the nephrin antibody showed staining of the germinal centres. Immunofluorescence staining of frozen sections of human adenoid revealed membrane-type staining of cells in the B lymphocyte (CD19+) areas. No nephrin positive cells could be seen in the T lymphocyte region. The staining pattern was characteristic for interdigitating follicular cells, as verified in the double-staining with nephrin and CD21 antibodies, a marker diagnostically used for detection of follicular dendritic cells. The specificity of these stainings was confirmed by blocking experiments.

5.3 Autoantibodies to nephrin, densin and filtrin in patients with type 1 diabetes (III, IV, unpublished)

The presence of autoantibodies in the serum of type 1 diabetic patients against shared molecules between kidney glomerular podocytes and pancreatic beta cells (nephrin, filtrin, densin) were studied by individual radioimmunoprecipitation assays.

5.3.1 Autoantibodies to nephrin (NAA) (III)

Altogether 43.9% of the patients with type 1 diabetes gave at least one sample positive for NAA, whereas none of the healthy control subjects showed positivity. At diagnosis, 24.2% patients tested positive for NAA and the respective proportions

at 2, 5 and 10 years were 23.0%, 13.8% and 18.2%. Only four patients (6.1%) tested positive in all measured samples. The levels of NAA fluctuated considerably over the 10-year measurement period.

5.3.2 Autoantibodies to densin (DAA) (IV)

Altogether 33.3% of the patients gave at least one sample positive for DAA, whereas only 2.2% of the healthy control subjects showed positivity ($p<0.001$). At diagnosis, 22.7% patients tested positive for DAA. The respective proportions at 2, 5 and 10 years were 8.2%, 13.8% and 9.1%. Of the 22 DAA positive patients, 14 had detectable densin autoantibodies at one of the time points, eight at two or three time points and none at all four time points, respectively. Similar to the established diabetes-associated autoantibodies (ICA, GADA, IA-2A), DAA positivity was typically detected already at diagnosis in most (68.2%) DAA positive cases. Furthermore, the DAA titres were the highest at diagnosis and decreased significantly already by 2 years of follow-up ($p<0.001$).

5.3.3 Autoantibodies to filtrin (FAA) (IV)

Altogether 10.6% of the patients had at least one sample positive for FAA, whereas only 2.6% of the healthy control subjects showed FAA positivity ($p=0.049$). At diagnosis, FAA were detected in 3.0% of the patients and the proportions at 2, 5 and 10 years were 4.9%, 5.2% and 6.8%, respectively. Of the seven positive patients, four had detectable FAA at one of the time points, three at two or three time points and none at all four time points. In contrast to DAA, FAA could appear at any time point during the 10-year observation period and FAA titres did not change significantly during the follow-up ($p=0.281$).

5.3.4 Clinical and genetic profile of the patients (III, IV)

The clinical data of the University of Oulu patient material at diagnosis was categorised by the presence or absence of NAA, DAA or FAA. The examination did not show significant differences in addition to the finding that DAA positivity was seen considerably less frequently among males than among females. 27.2% of

the patients were diagnosed with diabetic retinopathy during the follow-up period but no association was detected between the presence of NAA and the development of diabetic retinopathy.

The association between NAA, DAA and FAA and different HLA DR genotypes was also investigated. No significant association could be seen between DAA or FAA and various HLA DR combinations. However, the NAA and FAA positive patients always carried a HLA DR3 and/or HLA DR4 allele.

5.3.5 Immunological profile of the patients (III, IV)

The prevalence of NAA, DAA, FAA, ICA, GADA, IA-2A at different time points during the 10-year follow-up are presented in Figure 4. The prevalences of IA-2A and GADA closely followed the prevalence of ICA. The proportion of ICA (p=0.004), GADA (p=0.024) and IA-2A-positive patients (p<0.001) decreased from the time of diagnosis to the end of the follow up, and a similar trend was observed for DAA (p=0.010) but not for FAA (p=0.13). Furthermore, NAA titers did not correlate with ICA titers during the ten year follow-up.

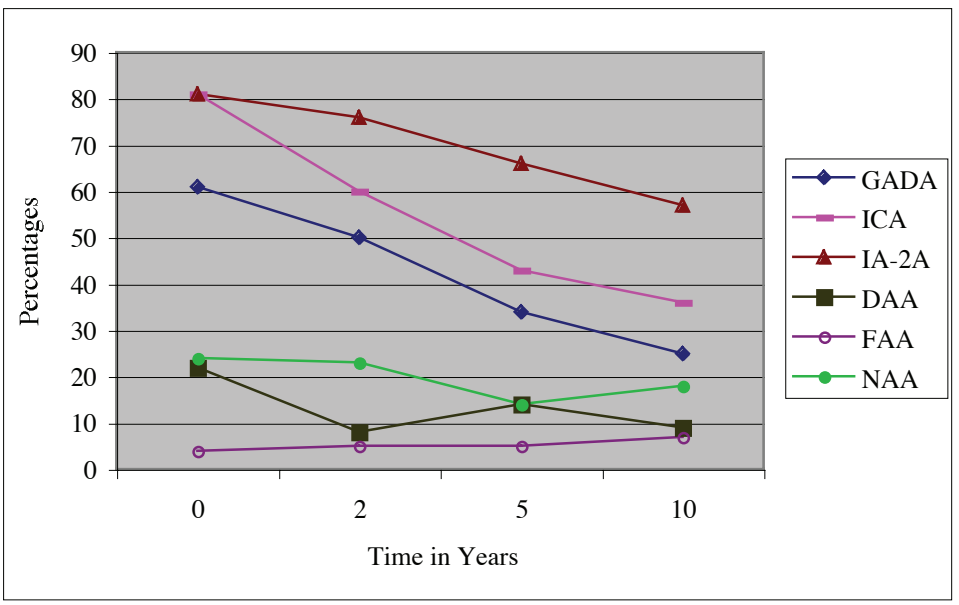

Figure 4. *Prevalence of the different autoantibody specificities during the ten year follow-up. The figure is modified from Publication IV by adding the NAA prevalence reported in Publication III.*

The possible association of DAA and FAA positivity with the positivity for the diabetes-associated autoantibodies at different time points was also assessed. An association could be detected between DAA and GADA prevalence at diagnosis (p=0.023) and at 10 years (p=0.045). Furthermore, the DAA positive patients had higher titers of GADA (p=0.001) and ICA (p=0.051) at diagnosis compared to the DAA negative patients. On the contrary, no association was seen between FAA and the diabetes-associated autoantibodies. The NAA, DAA or FAA were never detected alone, but rather they were always observed in patients in conjunction with other diabetes-associated autoantibodies.

In summary, NAA (24.2%), DAA (22.7%) and FAA (3.0%) have a low prevalence when comparing to either ICA (80.0%), GADA (61.5%) or IA-2A (80.0%) at diagnosis.

5.3.6 NAA, DAA and FAA in diabetic nephropathy (III, IV, unpublished)

The association between the novel autoantibodies (NAA, DAA, FAA) with diabetic nephropathy was studied using two separate patient materials; the University of Oulu patient material and the Finnish Diabetic Nephropathy (FinnDiane) study patient material. The patients in the FinnDiane material had had longer duration of diabetes and more symptoms of diabetic nephropathy compared to the University of Oulu patient material, thus the material was valuable in elucidating further the persistence of the autoantibodies and their association to diabetic kidney disease.

In the University of Oulu patient material, only a minority of the patients had developed signs of diabetic kidney disease. Among the 66 patients in this study, 14 (21.2%) had developed microalbuminuria during the total observation period of 17 to 19 years, and three of them (4.5%) had progressed to proteinuria. The mean duration of diabetes at the manifestation of microalbuminuria and proteinuria were 9.5 and 12.0 years, respectively. The time from diagnosis of diabetes to manifestation of renal injury varied considerably between the patients. Among the 14 patients with signs of renal injury, four (28.6%) tested positive for NAA at least at one of the time points. The corresponding numbers were four (28.6%) for DAA and two (14.3%) for FAA. In patients with NAA the mean duration of diabetes at manifestation of

microalbuminuria was 7.7 years whereas the patients free of NAA had a later manifestation of microalbuminuria at a mean of 10.1 years. However, this finding was statistically insignificant (p=0.42). All the three patients with proteinuria had DAA at one of the time points. No association could be detected between the FAA levels and manifestation of renal injury. In conclusion, no significant association of NAA, DAA or FAA to the manifestation or development of diabetic nephropathy was detected in the University of Oulu patient material.

A subsequent study was performed to investigate further the possible association of the expression of these novel autoantibodies to diabetic nephropathy. The type 1 diabetes patient material from the FinnDiane study was utilised for this purpose. The mean duration of diabetes in these patients was 22.6 years compared to the maximum of 19 years in the University of Oulu material. None of the FinnDiane serum samples, being either normo-, micro- or macroalbuminuric (thus being in different stages in the development of diabetic kidney disease), showed specific binding against nephrin, densin or filtrin autoantigens (unpublished results). The combined results from the University of Oulu and FinnDiane patient materials suggests that these novel autoantibodies are detected in a subset of patients with type 1 diabetes during the first ten years of clinical diabetes but are no longer detected in long-lasting diabetes.

5.3.7 Staining pattern of patient and healthy subjects sera (III)

It has been reported that autoantibodies against nephrin can be detected in CNF patient sera after kidney transplantation, and that a subset of CNF patient sera stain kidney sections in a glomerulus-like fashion in indirect immunofluorescence (Wang et al. 2001; Patrakka et al. 2002). Based on these findings we performed indirect immunofluorescence staining with diabetic patient sera to investigate if a similar phenomenon is detected. Immunofluorescence staining for rat kidney cortex sections was performed with the three diabetic serum samples with highest titers of NAA and three healthy control samples negative for NAA. An epithelial-like glomerular staining typical for nephrin was detected with two out of three of the highest serum samples and one of them showed no staining. Stainings with negative healthy control sera and secondary antibodies alone showed no specific reactivity.

6 Discussion

6.1 Podocyte proteins in the beta cells of the pancreas (I, IV)

In Publication I and IV, the podocyte molecules filtrin, FAT, alpha-actinin-4 and densin were found to be expressed in the pancreas, preferentially in the insulin-producing beta cells of the Langerhans islets. In addition, the expression of nephrin in the human pancreatic islet beta cells was confirmed, contradicting a recent report by Kuusniemi and colleagues (Kuusniemi et al. 2004). They studied nephrin expression at the mRNA and protein level in many extrarenal tissues of human and pig but they did not detect nephrin protein in any other tissue than the kidney. In the immunofluorescence studies they used an antibody against the whole intracellular part of nephrin as we did in study I. The contradicting results could be due to differencies in tissue collection protocols since it is known that pancreatic tissue requires extremely rapid tissue preservation to prevent any protein degradation. There could also be some differencies in our staining protocols which could affect the results. Zanone and colleagues have also studied nephrin expression in the pancreas but they suggest nephrin to be solely expressed in the endothelial cells of pancreatic islet microcirculation (Zanone et al. 2005). Based on our studies, the partially similar expression of these molecules in the podocytes and the beta cells suggests similarities in the molecular composition between these two cell types.

6.1.1 Structure of the hypothetical protein complex

Adherens junctions, the main components which are cadherins, regulate the adhesive properties of the beta cells (Rouiller et al. 1991). Cadherins are a family of ubiquitously expressed transmembrane cell adhesion molecules which mediate the homophilic cell-cell adhesion between neighbouring cells (Takeichi et al. 1988). Cadherins bind with their cytoplasmic tails to members of the catenin family, which link the cadherins to the cell cytoskeleton (Ozawa et al. 1989). The main cadherin of the pancreatic islet cells, the E-cadherin, has been shown to be

crucial for the proper organisation of the endocrine pancreas during development (Rouiller et al. 1991; Dahl et al. 1996).

In the present study, it was hypothesised on the basis of our novel findings of the podocyte proteins (nephrin, densin, filtrin, FAT and alpha-actinin-4) in the pancreatic beta cells, and the previous knowledge of the role of these molecules in other tissues, that these molecules participate in adherens junction-type cell-cell contacts between the beta cells comparable to those in the podocytes (Reiser et al. 2000).

There are many studies suggesting an interaction between these novel beta cell molecules and adherens junction molecules in tissues other than pancreas. An interaction between densin and a cadherin-family member has been shown in neurons and podocytes, specifically an interaction with N-cadherin in the neuron (Izawa et al. 2002) and with P-cadherin in the podocyte (Heikkilä et al, unpublished results). Since densin binds to both N- and P-cadherin, it probably can bind to the E-cadherin suggesting a similar interaction between an established adherens junction molecule and densin in the beta cells. Interestingly, nephrin has been found from the same protein complex with densin and P-cadherin in the glomerulus (Heikkilä et al, unpublished results), suggesting that nephrin could be associated with the same adherens junction-like protein complex. Furthermore, densin has been shown to directly interact with the actin bundling protein alpha-actinin-4 in the brain (Walikonis et al. 2001). The extracellular parts of NEPH1 (Barletta et al. 2003) and NEPH2 (Gerke et al. 2005) have been shown to interact with nephrin in the podocytes, and based on the homology, filtrin suggestively interacts as well. FAT, the large protocadherin found in the beta cells, has been suggested to be able to bind to catenins (Ponassi et al. 1999). Thus, the hypothetical protein complex formed by E-cadherin, densin, nephrin, filtrin and FAT could be linked to the beta cell cytoskeleton via both alpha-actinin-4 and/or catenins. Furthermore, nephrin could possibly act in a homophilic fashion to strengthen the cell-cell contact formed by E-cadherin between neighbouring cells.

6.1.2 Function of the hypothetical protein complex

What could be the implication of this suggested adherens junction-like cell-cell contact in

the beta cell? E-cadherin mediated cell adhesion between the islet cells has been shown to be crucial for insulin secretion (Cirulli et al. 1993; Yamagata et al. 2002), thus the hypothetical protein complex might have an indirect role in insulin secretion by maintaining the appropriate islet structure. Signaling processes may play a role in this process since many of these novel beta cell molecules (Huber et al. 2005) have been shown to have signaling function in addition to the signaling function shown for the cadherin-catenin complex (Wheelock et al. 2003). Interestingly, nephrin has been suggested to have a crucial role in insulin responsiveness in the novel insulin target tissue, the podocytes (Coward et al. 2005a; Coward et al. 2005b). There nephrin appears to be needed for the proper translocation of insulin independent glucose transporter molecule GLUT1 and insulin dependent glucose transporter GLUT4 to the plasma membrane (Coward et al. 2005a). GLUT2 in an insulin independent glucose transporter found from the beta cells, thus it can be hypothesised that nephrin would be needed for GLUT2 translocation in these cells. Hence nephrin and its associated protein complex may have an active role in glucose transporter translocation in insulin producing beta cells as well as in novel insulin sensitive cells, the podocytes, in addition to a suggested structural role in adherens-like junctions. Nephrin, however, does not appear to be crucial for the insulin secretion of the beta cells since no deficiency in glucose tolerance has been detected in CNF patients and CNF patients do not appear to be more prone to develop diabetes (Kuusniemi et al. 2004).

As a conclusion, nephrin could take part in GLUT2 translocation in pancreatic beta cells, and furthermore, it can be hypothesised that there could be a novel adherens junction–like cell-cell contact, formed by E-cadherin, nephrin, filtrin, densin and FAT in the beta cells. This junction could participate in the establishment and maintenance of the islet architecture and this way indirectly affect the ability of the beta cells to secrete insulin. Alpha-actinin-4 and/or catenins could link this whole complex to the beta cell cytoskeleton. This hypothesis could be tested by studying pancreas-specific interactions by co-immunoprecipitations and the exact localisation of these molecules in the pancreas by immunoelectronmicroscopy.

6.2 Nephrin in human lymphoid tissues (II)

In study II, nephrin expression was found in the lymphoid tissues, specifically in the germinal centres of the lymphoid follicles in the adenoids and lymph nodes. Nephrin was detected as a 165-kDa protein corresponding to the size of nephrin found in the pancreas (Palmen et al. 2001). This suggests different posttranslational modification, especially glycosylation, in the lymphoid tissues and pancreas compared to glomerular podocytes where the size is 185 kDa (Holzman et al. 1999; Ruotsalainen et al. 1999). Colocalisation with the CD21 antibody suggests nephrin expression specifically in the follicular dendritic cells (FDC) of the germinal centres. Since nephrin has a central structural role in the interpodocyte slit diaphragm, it may comparably participate in the maintenance of the lymphoid follicle structure in the lymphoid tissues.

In the lymphoid follicle germinal centre, the antigen-activated B cells rapidly expand and differentiate when they come into contact with FDCs, generating plasma cells and memory B cells. These cells have the capacity to produce antigen-specific antibodies. The FDCs maintain the framework of the lymphoid follicle and offer the B lymphocytes a stable network for proliferation and differentiation. The characteristic feature of FDCs is that they do not process and present antigens in HLA II molecules but rather present intact antigen-antibody complexes on their surface. The antigen-antibody complexes are held on the cell surface by Fc receptors or by complement receptors such as CD21. The contact between the germinal centre B cells and FDCs is crucial, since only those B cells that bind to immune complexes on FDCs surface can survive; the B cells which can not bind to the immune complexes are removed by apoptosis. If the regulation of B-cell apoptosis and elimination of self-reactive B cells is impaired, it results in autoimmune diseases (Park et al. 2005).

Germinal centre FDCs and glomerular podocytes appear to have some structural similarity since both of them have long extending cytoplasmic processes and cell-cell junctions between these processes from neighbouring cells. In addition to the frequent desmosome-like junctions between the processes (Schafer et al. 1996), adherens junctions with "classical" cadherins and beta-catenin have been suggested to exist in the

human FDCs (Muller et al. 2000). Furthermore, the formation of adherens junctions appears to be a prerequisite for the formation of desmosomes (Huber 2003). Since nephrin has been associated with adherens junction-like cadherin-catenin complex in the glomerulus (Lehtonen et al. 2004), it could have a similar association in the FDCs. Thus, nephrin could in a comparable manner to its function as a structural adhesion molecule in the slit diaphragm, act in the germinal centres as a adherens junction molecule and support the FDC network. In CNF patients (who lack functional nephrin), however, no dysfunction in B cell maturation or in antibody production has been reported, thus it can not be suggested that nephrin would be essential for the function of FDCs. Multiple infections are seen in patients with CNF but they have been suggested to be a consequence of antibody loss by proteinuria (Huttunen 1976; Hamed et al. 2001) rather than a defect in antibody production.

6.3 Novel autoantibodies in patients with type 1 diabetes (III, IV)

In studies III and IV, autoantibodies against novel beta cell molecules, nephrin, densin and filtrin, were found in a subset of patients with type 1 diabetes followed from the time of diabetes diagnosis for ten years. These autoantibody studies were based on the discovery of the novel beta cell molecules in studies I and IV, as well as on a report of nephrin expression in the pancreas by Palmen and colleagues (Palmen et al. 2001). Nephrin, densin and filtrin were selected in these autoantibody studies since they appear to have a very restricted tissue expression pattern, mainly in the kidney, the brain and the pancreas, as well as restricted expression in the pancreas itself, mainly in the beta cells of the Langerhans islets. For the autoantibody screenings, intracellular parts of these molecules were chosen as antigens, since in healthy individuals they are assumed to be protected in the cytoplasm, but during the immune destruction of the beta cells in type 1 diabetes these antigens can be released from the cytoplasm and induce a humoral immune response. Furthermore, since GAD and IA-2 both are intacellular molecules, it gave further justification for the selection of intracellular parts of nephrin, densin and filtrin as antigens. Humoral immune responses to various beta cell autoantigens, such as insulin, IA-2 and GAD have been shown to

associate with the early stages of diabetes.

GADA and/or IA-2A are present in more than 90% of the cases with recent onset type 1 diabetes (Savola et al. 1998a), and they are used as markers for type 1 diabetes. Compared to the established diabetes-associated autoantibodies (IAA, IA-2A, GADA and ICA), nephrin (NAA), densin (DAA) and filtrin autoantibodies (FAA) appeared to have a low prevalence at the time of diagnosis of clinical diabetes. The prevalence of NAA and DAA declined over time comparable to the prevalence of ICA, IA-2A and GADA, suggesting that NAA and DAA are also generated during the early autoimmune process leading to type 1 diabetes. DAA especially were typically detected already at the time of diagnosis in the diabetic patients and declined sharply thereafter. FAA was an exception to NAA and DAA since its prevalence did not decrease during the ten-year follow-up period and, furthermore, FAA could appear at any time point during the follow-up. This could, however, result from the fact that FAA were apparently expressed in lower levels than the other novel autoantibodies in the patient sera. Such low levels can easily be interpreted as a negative finding, especially since we chose a relatively high cut-off limit for positivity to avoid false-positive interpretations.

6.3.1 Mechanisms of autoantibody formation

At least two mechanisms could be responsible for the formation of these novel autoantibodies during the autoimmune process of type 1 diabetes: epitope spreading and loss of self-tolerance to specific molecules. It has been suggested that in those individuals who develop type 1 diabetes, islet cell autoimmunity starts as a non-pathogenic Th2 (T helper 2) response of the T helper cells, but develops into a pathogenic Th1 (T helper 1) response. The Th1 response is associated with epitope spreading (Katz et al. 1995; Petersen et al. 1999; Tuohy et al. 1999). Epitope spreading is a mechanism where the original autoimmune reaction against one epitope in one molecule is extended to other epitopes. Epitope spreading in detected within one molecule, eg. for GAD (Bonifacio et al. 2000; Sohnlein et al. 2000; Hoppu et al. 2004) and IA-2 (Naserke et al. 1998), but also between different molecules (Naserke et al. 1998). Thus, the development of autoantibodies

against nephrin, densin and filtrin in diabetic patients could be due to intermolecular epitope spreading from the original priming antigens in insulin, IA-2 and/or GAD to nephrin, densin and filtrin antigens.

Another possible explanation for the formation of autoantibodies againt nephrin, densin and filtrin could be the impairment in the development of self-tolerance to these molecules. The thymus has an ability to express small amounts of tissue-restricted antigens to be recognised by the developing T cells, which results in tolerance of the immune system to these antigens; if this is impaired it can result in an autoimmune reaction against these molecules (Derbinski et al. 2005). Impaired expression of insulin by the thymus has been suggested to be a factor in the development of autoimmunity in type 1 diabetes (Bennett et al. 1995; Pugliese et al. 1997; Vafiadis et al. 1997). An impaired expression of nephrin, densin and filtrin in the thymus could thus potentially explain the autoimmune reaction against these molecules. Interestingly, nephrin expression was detected in the thymus at the mRNA level in study II, which could represent the thymic expression needed for nephrin tolerance. One possibility to test this hypothesis could be to determine if nephrin expression is impaired in the thymus of patients with type 1 diabetes.

In summary, NAA, DAA and FAA were present in the serum of a subset of patients with type 1 diabetes, and they are suggestively a part of the common humoral autoimmune response seen in type 1 diabetes. This view is further supported by the finding that NAA, DAA and FAA were always detected in conjunction with the established diabetes-associated autoantibodies.

6.4 Novel autoantibodies in diabetic nephropathy (III, IV, unpublished)

In studies III and IV and in the following unpublished study, the possible association of NAA, DAA and FAA with the pathogenesis of DN was explored. The hypothesis that these novel circulating autoantibodies could be associated with the pathogenesis of DN was based on the following findings: a significant proportion of patients with CNF treated with renal transplants had circulating antibodies to nephrin (Wang et al. 2001; Patrakka et al. 2002) and an injection of monoclonal

nephrin antibody to rats resulted in massive proteinuria (Orikasa et al. 1988; Topham et al. 1999). It was also shown that the nephrin antibodies can reach the podocytes and bind to them (Orikasa et al. 1988). Interestingly, the staining of rat kidney sections with a type 1 diabetic patient sera with high nephrin autoantibody titer (study III) showed epithelial-like staining in the glomerulus. However, since no blocking experiments with nephrin antigen were performed, it cannot be argued that the staining was nephrin-specific.

In the studies performed with the University of Oulu patient material (studies III and IV), with a ten-year follow-up from the diagnosis of type 1 diabetes, NAA, DAA and FAA were found in a subset of patients with signs of DN, but no association was found between the autoantibodies and the development of DN; the patients developing kidney complications and those remaining unaffected had a similar prevalence of NAA, DAA and FAA. The University of Oulu patient material was not optimal in exploring the association of these autoantibodies to DN since the diabetes duration was only maximum for 10 years and only 14 patients out of a total of 66 patients had any signs of the kidney complication. Thus the study cohort may have been too small to assess the possibility that circulating antibodies induced by nephrin, densin and filtrin in the pancreas could end up in the kidney glomerulus and recognise the respective epitopes in the kidney. However, when the presence of these autoantibodies was studied in patients with longer duration of diabetes (average 22.6 years, unpublished study) and established symptoms of DN, no autoantibodies were detected. This finding strengthens the view that NAA, DAA and FAA are associated with the early stages of type 1 diabetes rather than with the later stages and late diabetic complications such as DN.

6.5 Conclusions and future prospects

In this thesis work, novel expression sites of podocyte molecules were shown, specifically densin, filtrin, FAT and alpha-actinin-4 in the pancreatic beta cells and nephrin in the lymphoid follicle germinal centres. The role of these molecules at these novel expression sites is proposed to be mostly a structural one, behaving as components of the adherens junction-like cell-cell contacts. Interestingly, all these molecules

appear to have a comparable tissue expression pattern since nephrin, densin, filtrin, FAT as well as alpha-actinin-4 have all been reported to be expressed in the renal podocytes, pancreatic beta cells and neurons of the brain. Further studies are needed to decipher the exact localisation and role of these molecules, and the structural and functional similarities of these three tissue types.

Autoantibodies against the novel beta cell molecules nephrin, densin and filtrin were found in a subset of patients with type 1 diabetes. However, no association could be shown between these autoantibodies and the development of diabetic nephropathy. It would be interesting to find out if these novel autoantibodies can be found in patients with LADA (the latent autoimmune diabetes is adults) and if they would be useful in the prediction or classification of this form of autoimmune diabetes. This would be especially interesting in the context of of densin autoantibodies since they were associated with GADA titers and GADA prevalence in the ten year follow-up material.

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9 Original publications